BIOCHEMICAL, NUTRITIONAL AND TOXICOLOGICAL STUDIES ON THREE VARIETIES OF *LABLAB PURPUREUS* (L.) SWEET SEEDS

BY

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ABSTRACT

Lablab purpureus is a lesser-known legume in Southwest Nigeria that could meet the protein requirement of people in the tropics. There is a need for investigation to ascertain its full potential. The biochemical, nutritional and toxicological effects of three varieties of *Lablab purpureus* seeds were investigated in male albino Wistar rats.

The three varieties of the seeds used were Rongai Brown, P_1 509 114 (RB), Rongai White, NAPRI 4 (RW) and Highworth Black, Grif 12293 (HB). Proximate composition, Antinutritional Factors (ANF) and protein quality were determined using standard analytical procedures. Sixty-five male albino Wistar rats divided into control, RB, RW and HB groups were used for the studies. Changes in the haematological, serum biochemical and histological parameters following *ad libitum* feeding of *L. purpureus* were used as indices of toxicity with standard biomarkers and analytical procedures. Biomarkers of oxidative injury to the liver, kidney and testes were also used to assess the level of toxicity. The control group received standard feed. The biochemical characterization of *L. purpureus* seed proteins was done using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Data were analysed using Analysis of Variance (ANOVA) and Student t-test at $p=0.05$.

The three varieties of *L. purpureus* contained crude proteins ranging from 22.8% to 24.2% and the crude fibre were appreciably high ranging from 12.7% to 13.1%. Trypsin inhibitors, haemaglutinnins, cyanogenic glucosides, oxalates, phytates, tannins, saponins and alkaloids were found in the three varieties of *L. purpureus*. All the rats placed on the three raw *L. purpureus* diets had low values of protein efficiency ratio, net protein ratio and protein retention efficiency. Compared with the control group, feeding the RB, RW and HB seeds for seven days produced significant reductions in the concentrations of red blood cells (5.0 ± 0.4, 6.5 ± 0.4 and $5.8\pm0.2\times10^{12}$/L), haemoglobin (9.0 ± 0.7, 11.6 ± 0.5 and 10.7 ± 0.2g/dL) and packed cell volume (30.4 ± 1.5, 35.4 ± 1.5 and 32.4 ± 1.8%) respectively. The RB, RW and HB seeds produced significant increases in the activities of serum alanine aminotransferase (74.5 ± 4.7, 40.8 ± 3.5 and 53.8 ± 1.9U/L), aspartate aminotransferase (266.5 ± 21.7, 173.5 ± 11.1 and 235.5 ± 10.1U/L), alkaline phosphatase (341.8 ± 35.4, 155.0 ± 2.9 and 177.0 ± 6.4U/L) and gamma glutamyl

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transferase (29.8±3.0, 13.8±1.6 and 21.5±0.6U/L). Similarly, there was an increase in urea (89.0±5.0, 45.7±1.5 and 65.5±3.9mg/dl) and creatinine (1.1±0.1, 0.5±0.1 and 0.8±0.1mg/dl) levels. Histology of the rat liver revealed mild congestion of hepatic blood vessels and severe diffuse hepatic necrosis. The kidneys showed moderate congestion of blood vessels with focal haemorrhages, while the testes showed degeneration and necrosis of seminiferous epithelial cells.

Lablab purpureus could be a good source of protein for poor countries of the world. However, it induced alterations in the hepatic, nephrotic and testicular antioxidant systems, impaired testicular and epididymal sperm function. Proper processing is necessary to reduce the antinutritional factors.

Keywords: *Lablab purpureus*, antinutritional factors, antioxidants, protein quality, toxicity.

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Now unto the living, eternal, immortal, invisible, the only wise God, be Honour and Glory for ever and ever. Amen.

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DEDICATION

This work is dedicated to:

1. My Lord and Saviour, Jesus Christ
2. The Memory of my Mentor and Former Supervisor, Late Professor M.A. Fafunso.
3. To the Memory of my Parents:
   Late Chief Albert Oluwole Soetan and Late Mrs. Esther Oyinlola Soetan.

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CERTIFICATION

I certify that SOETAN, KEHINDE OLUGBOYEGA carried out this work under my supervision.

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

The high cost of animal sources of protein has directed interest towards several leguminous seed proteins as potential sources of vegetable protein for human food and livestock feed (Esenwah and Ikenebomeh, 2008). Cheap and good quality sources of protein is the single most important limiting factor in animal nutrition in Nigeria (Bawa *et al.*, 2003). Conventional plant protein supplements in Nigeria are soybean meal and groundnut cake. However, they are very difficult to come by and are also very expensive, resulting in the high production cost for monogastric nutrition and a discouraging factor to farmers (Bawa *et al.*, 2003). In view of this, the use of underutilized legume grains having low preference as human food and which are obtainable at relatively lower costs are being investigated. Lablab beans is an example of such unconventional protein source and its nutritive value in livestock feeding has not been fully investigated (Bawa *et al.*, 2003). It is one of the lesser known legumes of arid and semi-arid regions and is classified by the National Academy of Science (NAS) as a potential source of protein that has not been explored (Osman, 2007).

In Nigeria and many other less-developed countries, protein-rich crop seeds are eaten in large quantities in compensation for the low consumption of animal protein (Adegbola, 1992). Most common plant protein sources for human consumption are also the major sources of plant proteins for the feed-formation of livestock. The resultant effects are the high cost of these conventional plant protein foods and a generally substandard feed production (Adegbola, 1992).

Malnutrition is widespread because of the deficiency of dietary proteins, particularly animal protein in human diets. This problem is the greatest challenge currently facing mankind. With the ever-increasing world population and the worsening food situation, the livestock industry has a key role to play so as to find or obtain the most economical source of protein. However, livestock in general, especially, rabbits, rats, poultry etc need grains and other sources of proteins like soyabean meal, fish meal, etc. These materials are used mostly in the industrialized countries; but sometimes in the

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tropical areas, they are limited in supply and are expensive. It is therefore very important to use other materials that are grown locally so as to reduce the demand for the conventional protein sources (Adegbola, 1992).

The major constraint to livestock production in Nigeria is the shortage of dry season feed, particularly shortages of forage and cheap sources of supplementary nitrogen (Onwuka, 1986). Breeding work through genetic improvement must be continued to incorporate desirable characters and improve the nutritional quality of forage legumes. The role of legumes, both as forage and as seeds and protein supplements in livestock feeding has been well reviewed (Tothill, 1986) and a number of them are now available for use in the tropical and subtropical areas. However, research activities have so far concentrated mainly on improved exotic species, while the abundant indigenous legumes remain underexplored. One such legume which is widely grown in Africa is *Lablab purpureus*. The bean can be used as seeds for human and livestock consumption (Pulsegrave, 1968, Skerman, 1977) and as forage for livestock (Hendriksen and Myles, 1980; Kiflewahid, 1986). In Tanzania, *L. purpureus* is 60-70% cheaper than soyabean. Although the bean is consumed by Tanzanians, it is not well liked by the people and is usually incorporated into their livestock diets There are very limited studies on the use of seeds generally as livestock feed (Sarwart *et al.*, 1991).

Locally available unconventional plant protein sources are rarely utilized either in human diets or as animal-feed ingredients because of the presence of anti-nutritional toxic components in these seeds (Aykroyd, 1982). Legumes are generally known to contain antinutritional factors (Ologhobo, 1980; Singh *et al.*, 1985; Ogun *et al.*, 1989). Liener (1989) reported the presence in soyabean of protease inhibitors, phytohaemagglutinins, goitrogens, phytic acids, amylase inhibitors, oestrogens, flatulents, allergenic substances, phenolic compounds, sterols and saponins.

Nature has deemed it fit to endow these plants with the capability to synthesize a wide variety of chemical substances which are known to exert deleterious effects when ingested by man and animals (Liener, 1983). Perhaps the best known of these toxic factors are the trypsin inhibitors which have the ability to inhibit the action of the enzyme trypsin found in the digestive tract of man and animals. The tremendous amount

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of work which has been done on these trypsin inhibitors stem from the important role the legumes have assumed in the fields of animal and human nutrition.

Apart from the trypsin inhibitors, the presence of substances in legumes which have the ability to agglutinate the red blood cells from various animal species has long been recognized (Ologhobo, 1980, Rajasekhar and Siva, 1997). These haemagglutinins are sometimes refered to as agglutinins or lectins. An attempt has been made to enumerate the toxic constituents of legumes because of the significant roles they play in human and animal nutrition and their anticipated roles in feeding the world of tomorrow (Gumn *et al*., 1983).

The fact that some sources of plant proteins are capable of producing harmful effects in animals is in itself of importance with respect to man's food supply. Were it not for the fact that such oil seeds as soyabeans and cottonseeds can be processed so as to inactivate their toxic constituents, these rich sources of plant proteins would not occupy the position of importance they now command in the feeding of animals and also in human food/diets.

This knowledge that food legumes contain anti-nutrients acquired from fertilizers and pesticides and several naturally-occurring chemicals made this research/study even more desirable. Some of these chemicals are known as "secondary metabolites" and have been shown to be highly biologically active (Zank, 1991). They include the saponins, alkaloids, tannins, phytates, oxalates and cyanogenic glycosides. Some of these chemicals have been shown to be deleterious to health or evidently advantageous to human and animal health, if consumed at appropriate amounts (Malinow *et al*., 1977; 1979; 1980; 1985; Malinow, 1985; Topping *et al*., 1980; Potter *et al*., 1990; Hardwood *et al*., 1993; Kersten *et al*., 1991; Sugano *et al*., 1993).

Most of these secondary metabolites elicit very deleterious biological responses, while some have found wide application in nutrition and as pharmacologically active agents (Oakenfull and Sidhu, 1989).

There is ample evidence that due to lack of nutrition education, most people consume badly prepared foods, which contain undetoxified heat-stable anti-nutrients that may be harmful to health. A handy example is cassava, known to contain cyanogenic

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glycosides (Osuntokun, 1970) and saponins (FAO, 1985). Food poisoning arising from plant secondary metabolites other than cyanogenic glycosides, has not been properly addressed in Nigeria, and indeed in most parts of the world that subsist mainly on legumes and vegetable-based diets. People have therefore died of ignorance and poverty, coupled with inadequate nutrition education and poor nutritional policies, especially within the African societies (Igile, 1996).

Plant toxicity causes both direct and indirect losses to the livestock industry and these effects have high economic consequences on livestock productivity. Indirect losses may result from reduced weight gains, decreased reproductive performance, fencing and management expenses (Adedapo, 2002). Some plant toxins may cause potentially hazardous residues in milk and meat. For example, trematone from white snakeroot (*Eupatorium rugosum*), selenium and several alkaloids may be passed in milk (Panter and James, 1990). Many instances of general malaise, anorexia and dullness in grazing animals which Veterinarians are called in to deal with, and the cause of which are seldom diagnosed, are due to the consumption of subclinical doses of some harmful plants (Clarke and Clarke, 1975; Abatan, 1992). Some of the toxic flora are yet to be scientifically investigated in Nigeria (Nwude and Parsons, 1977; Abatan, 1992). Most of the reported cases of plant poisoning in animal husbandry have been based on the observation of clinical symptoms after the animal might have consumed toxic quantities of the plant. For example, symptoms of bracken poisoning may not appear until months after the plant has been ingested (Blood and Radostits, 1989).

Some of these toxic plants occur generally around the country and some occur only in restricted areas in perhaps only one country. Some even well known quite deadly species in one region are used as feed in another region without any apparent effect (Abatan, 1992; Adedapo, 2002). The reasons for these variations in toxicity can sometimes be accounted for, sometimes guessed, and sometimes completely unknown. The differences in the degree of toxicity is said to be related to variations in growing conditions (Shaver *et al.*, 1964). Also, the presence or absence of a poisonous plant can be a local problem and a matter of the particular environment. Certain ecological conditions favour certain plant species. Variations in these conditions cause variations in

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Institute for Tropical Agriculture (I.I.T.A.) Ibadan. The pictures of the *Lablab purpureus* seeds and leaves are shown in Figures 1 and 2 below.

Figure 1: *Lablab purpureus* seeds

Source: Nwokocha *et al.* 2010.

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the prevalence of plants and in the likelihood of these plants poisoning the livestock. Such variations in plant toxicities therefore call for caution in trying to use the information from other areas to evaluate plant usefulness in other localities.

The plant used for this study is lablab beans (*Lablab purpureus*) and is available in Nigeria. It is fast gaining acceptability as a source of livestock feeds by Nigerian farmers and can also be easily accessible to grazing livestock and children at play.

The knowledge of the presence of active constituents in *Lablab purpureus* and the wide use of their seeds for food purposes in humans, and as feeds in animals, the use of its leaves and flowers as vegetables, forage and ornaments, and the medicinal use of the seeds and other outstanding qualities collectively necessitated this study.

Changes in haematological and biochemical parameters as well as clinical signs and histopathology were used as indices of toxicity because no literature in this part of the world has used these parameters as indices of toxicity for legumes generally. It should, however, be stated that these parameters are often used in toxicological evaluation of poisonous substances (Bush, 1991; Duncan *et al.*, 1994; Abatan *et al.*, 1996).

1.2 Plant Description

Lablab purpureus is an annual or short-lived perennial fodder legume grown for grazing and conservation in tropical environments. It is a vigorously trailing, twining herbaceous plant, resistant to disease and insect attacks (Milford and Minson, 1968; Cameron, 1988). Stems are trailing to upright, reach up to 3m in length and are robust. Leaves are large and trifoliate, with the leaflets having a broad ovate-rhomboid shape measuring 7 to 15cm long. The dorsal side of the leaf is smooth with the underside being hairy (Cameron, 1988).

Of the two hundred types of lablab so far recognized, only two varieties, Rongai and Highworth are available commercially (Cameron, 1988). Additionally, the three subspecies that have been identified so far are sub-spp purpureus, benghalensis and uncinatus. The three varieties used for this study are Rongai white (NAPRI 4), Rongai brown (PI509114) and Highworth black (GrIf 12293) obtained from the International

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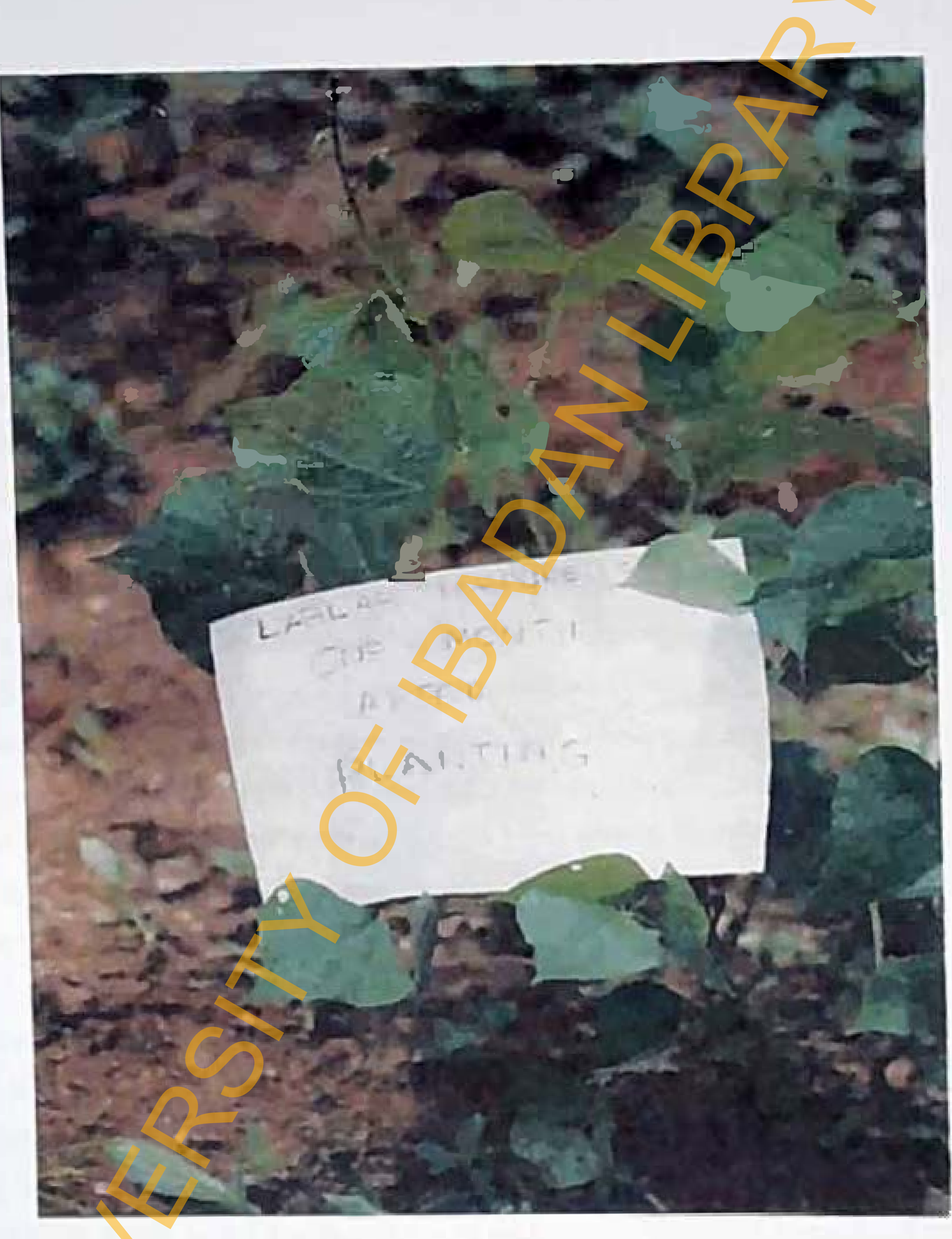

Figure 2: *Lablab purpureus* one month after planting

The wild forms of lablab are believed to have originated in India (Deka and Sarkar, 1990) and introduced into Africa from South-East Asia during the 18th Century (Kay, 1979). Presently, lablab is common in Africa, extending from Cameroon to Nigeria, Swaziland and Zimbabwe, through Sudan, Ethiopia, Uganda, Kenya and

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Tanzania (Skerman *et al.*, 1991). Currently, lablab is one of the major leguminous forage and green manure crop in this area of the world (Cameron, 1988).

Little is known about the domestication and the evolution of the lablab bean (Smartt, 1990), despite some debate about its origin (summarized by vonSchaaffhausen, 1963). Wild plants have been collected only from Africa (Verdcourt, 1970; 1979), whereas 'wild' plants obtained from India appear to be escapes from attempts of early cultivation (Smartt, 1990; Maass and Usongo, 2005). Based on a genetic study with AFLPs (amplified fragment length polymorphism), however, no convincing evidence was found to maintain that this crop had originated anywhere else than in eastern and/or southern Africa (Maass *et al.*, 2005). Despite its wide distribution in the tropics, it is still considered as neglected, in terms of research and development (Maass and Usongo, 2007). *L. purpureus* is associated with several names in different countries.

The English names of *Lablab purpureus* are hyacinth bean (American), bonavist bean, Indian butter bean, Egyptian kidney bean, tutuli bean. The French names are Lablob, dolique d'' Egypte The Kenya names are Njahi. Gambians: Manding-mandinka nalvo. Ivory Coast names are Anyi guangono (bean for enjoyment). Nigeria names: Hausa- "waake banbage" (bean of the pagans, "waake danfami" (fence bean). Igbo- "akidi fiofio", Yoruba- "ewa otili".

Culture

The plant is easily grown in poor acidic to alkaline soils taking 90-150 days from sowing to maturity. It needs full sun for best growth, moisture content, it requires well drained soil. Once established, lablab is drought tolerant than most bean. It is a short-lived perennial crop. The means of propagation is by seed.

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1.3 Already identified outstanding qualities of *Lablab purpureus*

i. It is easy to cultivate, very rich in nutrients and is quite palatable to ruminants (Ogundipe *et al.*, 2003).

ii. Lablab is recommended in Nigeria to solve the problem of inadequate feed provision for livestock, especially during the dry season (Agishi, 1983).

iii. Lablab is drought-resistant and grows well in the dry season when other plants serving as sources of animal feeds are dried up. It also maintains its nutritive value far into the dry season (*Adu et al.*, 1992).

iv. Lablab increases the liveweight and the milk-yield of cattle, when fed (Babayemi *et al.*, 2006).

v. Lablab has aggressive growth-habit, unlike other forages (Nworgu and Ajayi, 2005).

vi. In young pigs, it increases weight and feed consumption (Bawa *et al.*, 2003).

vii. In pullet chicks, it increases weight, feed consumption and feed-gain ratio (Ogundipe *et al.*, 2003).

viii. Lablab produces a high dry matter yield (Babayemi *et al.*, 2006).

ix. Lablab is one of the best sources of iron in ruminant feeds (Tropical legumes, 1979).

x. Legumes are generally highly susceptible to insect attacks both in the field and during storage (Caswell, 1981; Emechebe, 1981) but lablab is resistant to insect attacks, pests and diseases as it continues to grow vigorously in their presence (Cameroon, 1988; Flores, 1993).

xi. It is rapidly gaining acceptance by peasant and commercial livestock farmers (Ogundipe *et al.*, 2003) and looks promising as the legume of the future for both ruminant and monogastric nutrition.

xii. Lablab beans contain kievitone, a potential breast cancer-fighting chemical, not found in other legumes (Morris and Brad, 2003).

xiii. Lablab beans produced hypoglycaemic activity in alloxan-induced diabetic rats (Sharaf *et al.*, 1963; Pant *et al.*, 1968; Handa *et al.*, 1989).

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1.4 JUSTIFICATION FOR THIS STUDY

1. The nutrient and or chemical composition and the anti-nutritional factors of several legumes have been determined but data are scarce on similar information on *Lablab purpureus* seeds in this part of the world.
2. No comprehensive biological activity or feeding tests have been performed on the three varieties of *Lablab purpureus.*
3. As efforts are being geared towards improving the nutritive values of currently known legumes, efforts should also concentrate on the identification and assessments of nutritive use and toxic effects of the lesser known legumes, of which the lablab bean is one.
4. Data are scarce on the oxidant and antioxidant effects of antinutritional factors in legume seeds.
5. Data are scarce on the reproductive effects of antinutritional factors.

1.5 AIM and SCOPE of the Study

In describing the importance and role of toxic plants in our environment to both livestock and humans, it was emphasized by Clarke and Clarke (1975) and Hall (1977) that it is important to have an encyclopaedia of plant species which at one time or the other have been suspected to cause poisoning in livestock and or in man. Most of the reported cases of plant poisoning have been based on the clinical symptoms observed after the animals have consumed toxic quantities of the plant.

Little or no efforts have been devoted so far towards the biochemical evaluation and the mechanism of action of such toxic substances. Studies have shown that the growth depression caused by raw legumes are accompanied by a number of biochemical and physiological disturbances, but the extent to which each of these toxic substances contribute to the overall effect still remains obscure. Such studies are often useful for the development of tests to predict the risks, so as to facilitate the search for safer substances and the rational treatment of manifestation of toxicity (Gilman *et al.*, 1980).

The wide use of lablab seeds as food for humans, as animal feeds, as vegetable forage and as ornaments and medicinal components have collectively necessitated this

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study. The study was therefore designed to supply biochemical information on the three varieties of *Lablab purpureus* and to attempt to establish what relationship, if any, might exist between the biochemical parameters, antioxidants, reproductive effects, the pathological lesions and the anti-nutritional factors as they exist in the raw lablab beans. The work is also aimed at giving information on the nutritional and toxic effects of the seeds.

This study was therefore carried out to evaluate the nutritional and toxic effects of the three varieties of *Lablab purpureus* seeds using the following parameters:

1. Proximate composition comprising the % crude protein, % crude fat, % crude fibre, % ash, % Nitrogen-free extract, gross energy, % dry matter and % moisture. The Mineral Element Composition will also be analysed.
2. (a). Quantification of the antinutritional factors and toxic components including the estimation of the trypsin inhibitor units, haemagluttinating units, cyanogenic glycosides, oxalates, phytates, tannins, saponins and alkaloids.
   (b). Phytochemical screening of the lablab seeds.
3. Protein-Nutritional Quality Studies using some biological indices like Protein Efficiency Ratio (PER), Protein Retention Efficiency (PRE) and Net Protein Ratio (NPR).
4. Haematology: This is an essential part of the studies since some of the most dreaded side effects of poisons are haematological such as thrombocytopenia and red cell destruction. A complete blood count consisting of haemoglobin, packed cell volume, red blood cell count, white blood cell count and differential count will be determined for each animal.
5. Biochemical evaluations: These are important since biochemical changes are the earliest indicators of organic damage. Moreover, animals grazing on poisonous plants could be source of meat, milk or other edible by-products to man. Since it is the only way of observing such toxic effects while the animals are still alive, biochemical changes are thus useful. Such biochemical parameters include serum enzymes; alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-

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glutamyl transferase (γGT), urea, creatine, serum proteins (albumin and globulin).

6. Gross and histopathological lesions arising from organ toxicity, like liver, kidney and testes.

7. Studies on the antioxidant / free radical scavenging activities of the antinutritional factors including estimation of antioxidant enzymes like catalase, super-oxide dismutase (SOD), glutathione peroxidase, glutathione-S-transferase, hydrogen peroxide.

8. Molecular weight determination of the lablab seeds proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

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CHAPTER TWO

LITERATURE REVIEW

Food legumes represent a diverse group of plants. There are 13,000 species in the Order Leguminales, of which 18, including soyabeans, are characteristically grown for human consumption (Campbell-Platt, 1980). The use of legumes is particularly widespread in the tropics and subtropics (Srikantia, 1972). Legumes are important in human and animal nutrition in the less humid parts of the tropics, where they contribute substantially to the total protein intake, particularly in those less arid parts where the main energy is sourced from starchy roots and tubers, that contain little protein. In addition to their importance as food, legumes are important in agriculture as replenishers of soil nitrogen.

Legumes belong to a special family of plants botanically called leguminosae. They constitute a very large group of plants, second only to the cereals as a source of food for man and his animals (Allen and Allen, 1981). A wide variety of leguminous crops are found in the tropics, and include edible and non-edible species. The edible legumes include the Phaseolus species, which does very well in the tropical environment (Cobley and Steele, 1976). Legumes are found worldwide and are consumed in one form or the other, in practically every country of the world. About 20% of the protein currently available to man is derived from food legumes in the developing countries. Fermented foods derived from legumes and cereals are an important part of the human diet in Southeast Asia, the Near East, and parts of Africa (Allen and Allen, 1981).

According to Sinha (1977), legumes are used for a variety of purposes including: soil improvement, forages, vegetables, and ornamentals. Legumes are also known to contain antinutritional factors. Liener (1994) reported the presence in soyabeans of protease inhibitors, phytohaemaglutinnins, goitrogens, phytic acid, amylase inhibitor, oestrogens, flatulents, allergenic substances, phenolic compounds, sterols and saponins.

Fermentation has been shown to lead to improvements in the removal and digestibility of toxic substances, as well as reducing the cooking time (Whitaker, 1978).

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2.1 The Nutritive Value of Legumes.

The principal factors which determine the nutritive value of legumes as a source of protein are:

i. Its protein content in relation to total solids.

ii. The essential amino acid composition of the protein and.

iii. The availability of these amino acids for tissue metabolism.

Most dry legumes contain 20-25% protein and their amino acid patterns are comparable to those of animal protein, except that they are low in methionine and tryptophan (Stanton, 1966).

Most legumes have a low fat content, hardly exceeding 1-5% (Stanton, 1966). Their thiamine content varies between 1.12mg/100g in soybean to 0.53mg/100g in lima bean, 0.50mg/100g in the bambara groundnut, 0.89mg/100g in the cowpea and 0.50mg/100g in the pigeon pea. Since the human thiamine requirement is about 0.50mg/100g (FAO, 1962), legumes are adequate for human thiamine needs. Oyenuga (1968) further revealed that soybean contains 27.00mg/100g of ascorbic acid, 0.14mg/100g of riboflavin ad 17.4mg/100g of niacin. Cowpea contains 0.14mg/100g of riboflavin, 1.3mg/100g of niacin, while its ascorbic acid was too low to be detected. These values make legumes good supplements of diets in predominantly maize- eating areas where the disease, pellagra, prevails. It has been shown (FAO, 1968), that by adding 100g of beans to 300g of whole maize, protein intake was increased from 43 to 57g, niacin intake from 9.2-9.4mg arginine from 1.92mg/gN to 2.59, Histidine from 1.08 to 1.43mg/gN, lysine from 1.20 to 2.4mg/gN, tryptophan from 0.24 to 0.38mg/gN, methionine from 0.79 to 0.8mg/gN valine from 2.36 to 3.18mg/gN, leucine from 6.36 to 7.19mg/gN, isoleucine from 1.72mg/gN to 1.75mg/gN and calorie intake decreased from 1620 to 1603 calories. Vitamin A is also one of the food components known to occur in edible legumes. Soybean, cowpea, limabean, garden pea and pigeon pea contain varied amounts of vitamin A from 30 to 130 International Units/100g food. (Oyenuga, 1968; Stanton, 1966).

Besides the amino acids, carbohydrates, fats and vitamins, legumes are also good sources of minerals. Calcium is highest in the soybean with 220mg/100g, cowpea with

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90mg/100g, pigeon pea with 129mg/100g, kidneybean with 120mg/100g and lima bean 70mg/100g. Phosphorus has been reported to be 586mg/100g in the soybean, 451mg/100g in the cowpea, 280mg/100g in the pigeon pea and 7.6mg/100g in the bambara groundnut. No values were reported for the lablab bean and lima bean legume (Oyenuga, 1968). While the locust bean seems deficient in iron; cowpea, soybean, lima bean, pigeon pea, field bean, kidney bean and green gram have 4.00, 7.00, 6.00, 5.80, 4.60, 8.00 and 6.30mg/100g of iron respectively (Oyenuga, 1968).

From the above, it is obvious that the nutritive value of legumes is related to two factors: (i). The content of the essential nutrients and (ii).The availability of these nutrients. This second factor is very closely interwoven with the presence of a wide variety of chemical substances known to be toxic when ingested by man or animals, and which interfere with the digestive processes. Therefore, as legumes are the main potential protein sources for meeting the threatening protein shortage, the role of such naturally-occurring antiphysiological factors as they relate to their effects on animals, cannot be dismissed as being irrelevant to the problem of feeding the world of tomorrow.

2.1.1 Nutrient Composition of Legumes

The importance of legumes as food lies primarily on their high protein-caloric value. Early studies by Jones and Murphy (1924) revealed that legume-containing diets promoted growth in rats when supplemented with cysteine and when casein was substituted for the legume protein.

Sarwart *et al.* (1991) reported that, as the level of *L. purpureus* bean meal increased, there was a decrease in crude protein and an increase in crude fibre in the diets of broiler chicken fed 25% *L. purpureus* meal, but the changes were not significant ($P>0.05$). The beans were well accepted by the birds and the protein appeared to be well utilized, with a feed:gain ratio of 3:2. Babayemi *et al.* (2006) reported that feeding lablab at 50% supplementation with *Panicum maximum*-diets could lead to improved feed intake, weight gain, nutrient digestibility and nitrogen utilization in West African dwarf goats. Bawa *et al.* (2003) reported that 20% lablab seeds can be included in the diet of weaning pigs without any adverse effect on their performance characteristics.

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2.1.2 Protein

Legumes are nutritionally important because of their relatively high protein content among vegetable foodstuffs. The protein of grain legumes contains relatively more of the essential amino acids lysine and tryptophan and usefully complement the amino acid supplied by cereals in which the content of lysine and tryptophan are relatively small (Patwardhan, 1962). n the other hand, the proteins of legumes contain relatively small proportions of the sulphur-containing amino acids, methionine and cysteine (Patwardhan, 1962). The special value of legumes as food has always been appreciated, but it has recently been emphasized as a result of increasing world populations, and of the awareness of the need to produce more food, especially protein-rich foods. Their protein levels (except soybean) vary between 20 and 30% (Patwardhan, 1962), who also reported the characteristic features of legume proteins as being a deficiency in methionine and cysteine.

However, McDonald *et al.* (1981) reported the quality of legume protein to be a reflection of their high lysine content which is also similar to that of fish meal. Variations in protein content of legumes are known to be influenced by varietal and environmental differences (Swaminathan and Jain, 1973). Bressani *et al.* (1978) reported that the methionine content of *Pisum sativa* increases when the legume grain was fertilized with sulphur. Increased protein efficiency ratio (PER) is observed when soybean is used in supplementing lime-treated corn.

The essential amino acid composition of *Dolichos lablab* as reported by WHO/FAO (1965) is shown in Table 1 below.

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Table 1 Essential amino acid composition of *Dolichos lablab* (WHO/FAO, 1965).

Amino acid	g/16gN
Lysine	5.50
Methionine	0.80
Cystine	0.51
Phenylalanine	4.80
Tyrosine	2.18
Leucine	8.80
Isoleucine	4.40
Valine	5.81
Tryptophan	0.80
Threonine	3.50

2.1.3 Fat and Oil

Most legumes are poor sources of fat and oil, although groundnut, soybean and lucin are rich in them. Hilditch and Williams (1964) reported that legume seed oils contain oleic, linoleic, arachidonic, valeric, linolenic and palmitic acids. The proportion of these acids present vary widely in the different species of legumes (Oyenuga, 1966).

2.1.4 Vitamins

Some legumes contain carotene and the carotenoids but they are all good sources of thiamine, riboflavin and niacin (Stanton, 1966). The thiamine content varies between 1.12mg/100g in soybean and 0.53mg/100g in limobeans, 0.5mg/100g in bambam groundnuts and 0.5mg/100g in the pigeon pea. Since, the human thiamine requirement is about 0.50mg/100g (FAO, 1969), legumes are adequate for human thiamine needs. Oyenuga, (1968) reported that soybean contains 27.00mg/100g of ascorbic acid, 0.14mg/100g of riboflavin and 17.4mg/100g of niacin. All these values make legumes a good supplement of diets in predominantly cereal eating areas where the disease pellagra is very common. The most important vitamins in cowpea seeds are thiamin, riboflavin, niacin and folic acid (Ogunmodede and Oyenuga, 1968; 1969; 1970; Edijala, 1980 a, b) Cowpea and soyabeans contain more thiamin, riboflavin and niacin than whole milk and

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cereals, with the levels of these vitamins being comparable to those available from fish, beef and eggs (Elegbede, 1998).

2.1.5 Minerals

Legumes are good sources of minerals. Calcium is highest in the soybean with 220mg/100g, pigeon pea with 129mg/100g and limabean 70mg/100g. Legumes are a good source of zinc, phosphorus, magnesium and potassium. These minerals are important for maintaining healthy muscle tone, combating fatigue and promoting energy and endurance. Legumes are also a good source of iron which helps to deliver oxygen to all cells and of the B vitamins which are important for a number of bodily functions including the maintenance of healthy nerve cells and strengthening the immune system (Edijala, 1980 a, b; Watson, 2004). Cowpea, soybeans and bambara groundnuts are good sources of calcium and iron with their contents being higher than those of meat, fish or eggs.

Meiners *et al.* (1976) examined raw and cooked dry legumes for nine mineral elements and concluded that raw legumes are relatively high in calcium, iron, magnesium, phosphorus and potassium, but low in sodium. Oke (1987) reported that Nigerian pulses are poor in calcium, but contain relatively high amounts of magnesium and phosphorus. However, it is well known that not all minerals present in legume grains are necessarily available to the body. Nelson *et al.*, (1968) attributed the non-availability of calcium in legume-based diets to the inherent phytic acid content.

2.2 CONSUMPTION OF LEGUMES

Some varieties of legumes are eaten even before they mature, either the entire green pod is cooked, or the tender seeds are removed from the pod, cooked and eaten. Foods can supplement each other to give a higher protein value, because if there is a shortage of one amino acid in one food, it can be made up by another food containing this amino acid in excess. Cereal proteins are relatively deficient in the amino acid lysine but are rich in methionine and cystine while legume proteins are rich in lysine but are deficient in the latter. Tejado (1962) demonstrated that maximum protein efficiency ratio (PER) of a mixture of maize and beans is achieved when 50% of the total protein of the mixture comes from corn and 50% from beans. Such diets based on all vegetable

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proteins have been successfully used in child feeding programmes and are found to be very useful in curing some protein-deficiency diseases.

2.3 FACTORS AFFECTING THE UTILIZATION OF LEGUMES

Despite the nutritional asset of legumes, their utilization are known to be affected by the presence of toxic antinutritional factors (Ologhobo and Fetuga, 1983). These factors include trypsin inhibitors, haemagglutinins, tannins, phytates, oxalates and saponins (Liener, 1980). In addition to these antinutritional factors, legumes have also been reported to contain a cyanogenic glycoside which on hydrolysis yields toxic hydrocyanic acid (Ologhobo and Fetuga, 1984a).

2.4 CLASSIFICATION OF ANTI-NUTRITIONAL FACTORS

Anti-nutritional factors in plants relevant to foods and feeding-stuffs may be classified on the basis of their chemical structure, the specific actions they bring about, or their biosynthetic origin. They are quite numerous and form a highly diverse group of compounds (Table 2). Although this classification does not encompass all the known groups of anti-nutritional factors, it does present an array of those frequently found in foods and feeding stuffs.

Often confused with anti-nutritional factors are the so-called adventitious toxicants. Whereas the former are produced endogenously by, or within, living tissues, the latter are present in foods or feedingstuffs due to human activities such as processing, storage, packaging or transportation. The adventitious toxicants (as distinct from anti-nutritional factors) have been described by Friedman and Shibko, (1969) as unwanted contaminants which are derived from careless handling, spoilage or other uncontrolled circumstances. Like anti-nutritional factors, adventitious toxicants are physiologically active although often more thermostable. Quite often, low levels may be present in foods and feedingstuffs without deleterious effects. However, chronic exposure to such low levels of intake may eventually become hazardous.

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Table 2: Some Anti-nutritional Factors in Foods.

Parent compound	Distribution	Examples
1. *Proteins*		
Enzyme inhibitors	Most legumes, egg white.	Trypsin and chymotrypsin inhibitors, amylase inhibitors, elastase inhibitors, plasmin inhibitors.
Haemagglutinins	Most legumes.	Concanavalin A, ricin.
Enzymes	Soyabeans, lentil.	Urease, lipoxygenase.
2. *Glycosides*		
Cyanogens	Lima bean, cassava, sorghum, forage species etc.	Phaseolunatin, dhurrin, linamarin, lotaustralin.
Goitrogens	Legumes, brassicas.	Glucosinolates, pro-goitrins.
Oestrogens	Most legumes, some grasses.	Flavones, genistein.
Saponins	Legumes, solanum species, grasses.	Soya sapogenin.
3. *Phenolics*		
Gossypol	Gossypium species (cotton).	
Tannins	Most legumes, grasses, leafy vegetables.	Condensed and hydrolysable tannins.
4. *Amino acid analogues*	Lathyrus, Vicia, Leucaena, cycas.	BOAA, DAP, mimosin, α-N-methyl-l-alanine, canavanine.
5. *Alkaloids*	Lupins, potatoes.	α-Solanine, chaconine.
6 *Miscellaneous*		
Anti-metals	Most legumes, cereals.	Phytates and oxalates.
Anti-vitamins	Most legumes, cereals.	Anti-vitamins A, D, E, B_{12}
Favism factors	*Vicia faba*.	Vicine, convicine.

Source: Aletor (1993)

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2.5 NUTRITIONAL, BIOCHEMICAL AND PHYSIOPATHOLOGICAL EFFECTS OF ANTI-NUTRITIONAL FACTORS.

2.5.1 The Haemagglutinins [Lectins]

It has long been established that many members of the Leguminosae and Euphobiaceae, which are important dietary components of a large segment of the world's population, possess little nutritive value and may in fact be toxic, unless subjected to some form of heat treatment (Liener, 1969; Aletor and Fetuga, 1984 a – d; Aletor and Fetuga, 1985; Aletor and Fetuga, 1986; Aletor, 1993a)). One well-known example of these toxic factors is haemagglutinin; a protein with the remarkable property of agglutinating the red blood cells and precipitating polysaccharides and glycoproteins. The haemagglutinins are known to agglutinate malignant cells and induce mitosis in lymphocytes (Sharon and Lis, 1972).

Evidence that part of the toxic action of raw or improperly processed legumes is related to the presence of the haemagglutinin came from Liener and Palansch (1952), who indicated that a growth depression of about 75% relative to that of the control was obtained in the rat by the addition of soyabean haemagglutinin at 1% of a diet containing autoclaved soya bean. Studies with soyabean and other legumes (Pusztai *et al.*, 1979; Aletor and Fetuga, 1988a, b) have shown that ingested haemagglutinins depress growth in animals by interfering with the digestion and absorption of nutrients in the gastrointestinal tract. For example, dietary lima bean haemagglutinin at levels of 0.015 – 0.125% have been shown to cause growth retardation as well as a marked decrease in the digestibility of protein, starch and disaccharides in the rat (Aletor, 1987a).

Physiopathological changes have been induced in animals administered the hemagglutinin of castor bean (*Ricinus communis*), soya bean (*Glycine max*), kidney bean (*Vicia faba*), and lima bean (*Phaseolus lunatus*). These include increase in blood concentration of urea, glucose, bilirubin, transaminases and lactate, glutamate and isocitrate dehydrogenases (Ikegwuonu and Bassir, 1977; Aletor and Fetuga, 1984; 1985, 1988b; 1989). While information on the effects of dietary haemagglutinins on gut enzymes has remained scanty, studies by Aletor and Fetuga, (1988b); Aletor and Fetuga, (1989) indicate that they considerably alter the activities of these enzymes. Inhibition of

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brain acetylcholinesterase (AchE) activities by dietary lima bean haemagglutinin has been reported in the rat (Aletor & Fetuga, 1984c; Aletor, 1986 a), while pathological lesions in animals injected intra-peritonealy with kidney bean and castor bean haemagglutinins have been reported. Focal necrosis and fatty changes have been observed in the liver (Sharon & Lis, 1972; Ologhobo & Fetuga, 1986) while the kidneys and myocardium showed distended capillary vessels with numerous thrombi following toxic doses of certain bean haemagglutinins. Similarly, pathological observations have been reported in rats fed heamgglutinin-containing lima bean [Aletor, 1987b). The binding of haemagglutinins to intestinal cell membranes is believed to impair the normal absorption of dietary nutrients when foods or feeding stuffs containing these factors are consumed.

2.5.2 The Trypsin (Protease) Inhibitors

The realization that these anti-nutrients are widely distributed among important sources of dietary plant protein throughout the world has stimulated a vast amount of research on their nutritional significance. For example, it has long been observed that unless soya bean or, indeed, several other grain legumes are properly cooked, they do not support the growth of rats and chicken (Aletor and Otonimoyo, 1992; Aletor and Egberongbe, 1992; Aletor and Osungwu, 1992; Aletor, 1993b). Other experiments showed that the addition of methionine or cystine to unheated soya bean improved its utilization almost to the same extent as proper heating. It has been demonstrated that the physiological effect of trypsin inhibitors is to cause enlargement of the pancreas followed by secretion of excessive amounts of pancreatic enzymes, much of which is lost to the animal in faeces. In this regard, Liener (1976) and Abbey *et al.* (1979) showed that the mechanism whereby pancreatic hypertrophy can lead to growth depression in animals fed trypsin inhibitors is to cause dietary sulphur-amino acids (methionine and cystine) to be diverted from the synthesis of body tissues to the synthesis of pancreatic enzymes, which are relatively rich in these amino acids. This loss of sulphur-amino acids may in fact further accentuate an already critical situation with respect to the methionine and cystine content of most legumes. Evidence suggesting that the trypsin inhibitors in raw bean may not be completely responsible for

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the magnitude of pancreatic enlargement and hence the secretion of excess pancreatic enzymes has emerged from a study of the major limo bean anti-nutritional factors by Aletor and Fetuga, (1988a,b; 1989b). In the study, it was demonstrated that while raw lima bean caused a 71% increase in the weight of the pancreas, lima bean trypsin inhibitor fed at the same level of activity as in the raw bean caused a 33% weight increase. These results suggest that the trypsin inhibitors may be more potent in their unextracted forms, or that they may combine additively or synergistically with other factors in the bean when fed.

2.5.3 The Cyanogenic Glucosides

Cyanide is almost ubiquitous, although present in trace amounts, in the plant kingdom. Certoin forage species, root crops, grains, pulses and fruit kernels contain high levels of cyanide in the form of cyanogenic glucosides. The glucosides found in the highest concentrations include linamarin (as in cassava and flax) and phaseolunatin (as in lima bean or other pulses), while amygdalin, dhurrin and lotaustralin are commonly found in sorghum and other grasses used as fodder. It is generally believed that the toxic properties associated with linamarin and other cyanogenic glucosides are due to hydrocyanic acid (HCN) released from the glucosides by some enzyme complex.

Cyanide, either in synthetic inorganic forms as in KCN or NaCN, or organic forms as in cyanogenic glucosides, is o potent specific and non-specific inhibitor of several enzyme-catalysed processes (Aletor & Fetuga, 1988 a,b). For example, Tewe *et al.* (1977); Tewe and Maner (1980); Aletor (1993c) demonstrated that cassava diets with appreciable levels of cyanide cause marked changes in weight gain, apparent nitrogen digestibility and thiocyanate concentration in the urine and serum of rats and pigs. It is well established that the major pathway of cyanide detoxification in man and animals is through the cyanide-thiocyanate sulphur transferase (rhodanese) enzyme pathway (Osuntokun, 1972; Oke, 1973).

This route requires an organic sulphur-donor for the formation of the less acutely toxic thiocyanate from cyanide. The sulphur amino acids (methionine & cystine) are mainly used in cyanide detoxification, thus reducing the quality of the protein available for body growth. In fact, Investigations by Maner and Gomez (1973) have suggested that

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detoxification of cyanide by animals can condition methionine deficiency in an otherwise balanced diet.

While acute toxicity and death arising from the consumption of cassava and its products is not a common occurrence, many studies indicate that cyanide toxicity may arise from exposure to sub-lethal dietary doses. The prevalence of human ataxic neuropathy and of goiter is greater in cassava or other linamarin-containing products. There have been indications (Osuntokun, 1972) that the neuropathy is caused or aggravated by cyanide, and that thiocyanate, an established goitrogen, is a byproduct of ingested cyanide. There are reports of lesions in the central nervous system of rats injected repeatedly with sublethal amounts of cyanide. These include the degeneration of neurons in the cortex and corpus callosum of the brain. Demyelination of the optic nerves and retina has also been observed in rats chronically exposed to sublethal dietary cyanide. A study by Tewe (1975) showed that pigs fed sweet cassava diets containing 250 or 500ppm cyanide (as KCN) had high level of serum and urinary thiocyanate, which caused pathological changes in the thyroid glands of the pregnant gilt and the passage of thiocyanate through the placenta barrier to the fetuses.

Many plant species contain hydrocyanic acid (HCN) either free or more usually in the form of cyanogenic glycoside, an organic compound containing a sugar and capable of yielding cyanide on hydrolysis (Fernando, 1987). The glycoside itself is reported not to be poisonous but becomes so when the hydrocyanic acid content is released by an enzyme. The release of such enzyme is associated with the plant damage or decay (Herrington *et al.*, 1971; Fernando, 1987).The observation of hydrocyanic acid from lablab seeds may be an indication that among other chemical substances they contain,they also posses cyanogenic glycoside. According to Clarke and Clarke (1975), the minimum lethal dose of free hydrocyanic acid and of potassium cyanide given per os is about 2.0 to 2.3mg/kg HCN. In fact, it is reported that materials containing over 20mg HCN per 100g is potentially dangerous to livestock However, most Authors working on cyanide poisoning believe that it is not possible to state with any certainty the toxic dose of cyanide in form of the cyanogenic glycoside.This is because the level of cyanide varies according to the conditions obtaining in the plant and that of the animal at the

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time of consuming the plant (Clarke and Clarke, 1975). Cyanide concentrations are highest in the young actively growing plant. It has been reported that poisoning in ruminants depend upon the quantity of the plant ingested, the previous diet of the animal, the pH of the stomach contents, the percentage of the total hydrocyanic acid present in the free state in the plant, the concentration of cyanide liberating enzyme present in the plant and the total hydrocyanic acid content of the plant. It is also reported that only animals that rapidly eat the plants that die. It is also observed that ruminants are more susceptible to poisoning by cyanogenic plants than are horses and pigs, since the enzyme concerned in the release of hydrocyanic acid are destroyed by gastric hydrochloric acid (Clarke and Clarke, 1975). Moreover, hydrogen cyanide is detoxicated by conversion into thiocyanate and is excreted in the urine over a period of several days.

Due to the rapid detoxification of cyanide, it is reported that it is possible for animals to ingest amounts of cyanide only slightly less than the lethal dose over extended periods without harm (Hazzard *et al.*, 1982). In this situation, these seeds under study could be consumed over prolonged periods with their cyanide content not constituting danger.

2.5.4 Tissue response to cyanide

Cyanide is believed to exert its toxic effect by inhibiting the respiratory enzyme cytochrome oxidase. Concentration of cyanide as low as 3×10^{-8}M have been shown to produce complete inhibition of cytochrome oxidase *in vitro* and lethal doses of cyanide have been reported to inhibit this respiratory enzyme *in vivo* (Hazzard *et al.*,1982;Tewe and Manner, 1982; Tewe and Pesu, 1987). In cyanide poisoning, O_2 transport and O_2 tension are usually adequate and only cellular utilization of O_2 is depressed. As a result of this, administration of O_2 would not serve any useful purpose in antagonizing cyanide intoxication (Way *et al.*, 1966).

Significant amounts of cyanide can be detoxified in the body. The proposed routes of detoxification include conversion to thiocyanate ion (SCN^-), incorporation into the 1-carbon metabolic pool through interaction with vitamin B_{12} and conversion to 2-imino-4-thiozolidine carboxylic acid (Casarrett and Doull, 1975; Gilman and Goodman, 1980). It was proposed that an enzyme from the kidney and liver called rhodanase

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catalyses the reaction of thiosulphate with hydrogen cyanide (HCN) to give this cyanate,a relatively less toxic substance which can be rapidly excreted. The properties of the enzyme rhodanase, now known as thiosulphate S-transferase and of a second enzyme mercaptopyruvate S-transferase,also capable of forming thiocyanate by sulphur transfer to cyanide have been reviewd by (Sorbo, 1975). Wokes and Picard (1955) also suggested the involvement of vitamin B_{12} in cyanide detoxification. In this pathway, vitamin B_{12} in the form of cobalamine (B_{12a}) reacts with cyanide to form cyanocobalamine (B_{12}).The latter then looses some of the cyanide to form 1-carbon fragments for the synthesis of methyl groups and the resulting hydroxocobalamine returns to the liver to repeat the cycle. Also, the hydroxocobalamine can combine with the thiocyanate formed via the thiosulfate or mercaptopyruvic acid and proceed to form cyanocobalamine. Acute toxicity and death from consumption of cyanide-containing food in human, for example cassava and of plant materials in animals is a common occurrence. However, it is important to note that chronic effects from consumption of sub-lethal amounts of cyanide over long periods has been associated with occurrence of human ataxic neuropathy and also goitre in Nigeria. This has been observed in areas where consumption of a staple food is cassava (Osuntokun, 1973). It is suggested that the neuropathy is caused by or aggravated by a cyanide or its derivative acting on the central nervous system (CNS) and the thyroid enlarged by thiocyanate which is a well known goitrogen derived from metabolism of ingested cyanide (Osuntokun, 1972 and Wilson, 1973).

Animal experiments confirm such theories. Lesions in the CNS were observed when rats were injected repeatedly with sublethal amounts of cyanide over a period of 22 weeks, with some suggestions of demyelination (Smith *et al.*, 1963). Similarly, the injection daily for 5 weeks of potassium cyanide (KCN) in rats produce degeneration of neurons in the cortex and the degeneration of the corpus callosum (Smith and Puckett, 1965). Demyelination of the optic nerves and retina were observed in rats given sublethal doses of cyanide over periods of 3 weeks (Wessel, 1971).

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2.5.5 Phytic Acid

Phytic acid also known as myo-inositolhexaphosphate is an important constituent of certain legumes, cereals and forage plants and is capable of chelating divalent cationic minerals like calcium, iron, magnesium and zinc. Such chelates many elements making them nutritionally unavailable, thereby inducing dietary deficiencies (Nelson *et al.*, 1968). Phytic acid reduces the absorption of calcium from the gastro-intestinal tract and consequently implicated in the development of rickets when chicks are fed cereals such as sorghum. Zinc and iron deficiency symptoms have been reported in man and chicken (Lease, 1966) when fed diets high in phytic acid. There is a significant inverse relationship between phytic acid content and the availability of calcium, magnesium, phosphorus and zinc in products like soya bean, palm kernel, rapeseed and cottonseed meals (Nwokolo and Bragg, 1977). It is well established that unheated soya bean or its protein isolate in the diet of chicks can cause rickets, unless large doses of vitamins D3 are included in the diets. (Rackis, 1974; Maga, 1983). The thermolabile rachitogenic agent has since been identified as phytic acid.

Phytic acid can react with proteins to form phytate-protein complex which incorporates about 16% of the protein in beans. The reaction between phytic acid is influenced by the pH of the medium. Formation of a phytate-cation-protein complex is believed to account for the decreased mineral bioavailability in phytic acid. Apart from decreased mineral and protein bioavailability associated with the consumption of phytin-rich foods, it is also associated with increased cooking time in most grains legumes.

Extensive research has been conducted on the chemistry of phytic acid (Cheryan, 1980; Cosgrove, 1980) and its nutritional significance (Edman, 1979; Weingartner, 1981). Phytic acid may decrease the availability of divalent cations such as calcium, zinc and iron, by the formation of an insoluble protein-phytic acid- mineral complex, it has been cited as causing reduced availability of zinc in soybean foods (O'Dell and Savage, 1960; Forbes and Parker, 1977).

2.5.6 The Tannins

The tannins are complex polyphenolics found widely in the plant kingdom. Their concentrations are significant in millet, sorghum, barley and some leguminous

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forages and in leafy vegetables (Aletor, 1991a; 1993a; Aletor and Fasuyi, 1997; Agbede and Aletor, 1999).

Tannins bring about their anti-nutritional influences (especially in the monogastric animals) largely by precipitating or binding dietary protein and digestive enzymes to form complexes which are not readily digestible. The poor palatability generally associated with high-tannin diets can be ascribed to its astringent property (or sharp taste), which is a consequence of its ability to bind with the proteins of saliva and the mucosal membrane of the mouth during the mastication of food. Similarly, tannins strongly inhibit digestive enzymes and bind proline-rich proteins in the saliva. It is believed that iron absorption is inhibited by tea due to the formation of iron tannate while studies by Radhakrishna and Sivaprasad (1980) showed that high dietary tannin lowered iron availability in anaemic subjects. Feeding chickens with 0.5% tannic acid have shown growth depression, while death from higher dietary levels are not uncommon. Raspeseed meal from which tannins had been extracted has been shown to have a higher metabolizable energy density than the unextracted meal (Yapar and Clandinin, 1972). Ruminant animals generally have a higher capacity for dietary tannins than the monogastrics.

Tannins exist primarily in condensed (CT) and hydrolysable (HT) forms (Haslam, 1989). The HT molecule contains a carbohydrate (generally D-glucose) as a central core. The hydroxyl groups of these carbohydrates are esterified with phenolic groups such as gallic acid and ellagic acid (Haslam, 1989). The HT occur mainly in fruit pods and plant galls and unlike CT their degradation products are absorbed from the small intestine of animals (McLeod, 1974) and are potentially toxic to ruminants (Dollahite *et al.*, 1962). The CT or proanthocyanidins (PA) are the most common type of tannins found in forage legumes, trees and shrubs, and HT rarely occur in forages. However, HT are often present in leaves of trees and browse shrubs in tropical areas. Structurally, CT are complexes of oligomers and polymers of flavonoid units (i.e. flavan-3-ols, flavan,3,4-diols and biflavans) linked by carbon-carbon bonds (Hagerman and Butler, 1991), they normally occur in cell vacuoles. CT are of interest in ruminant nutrition because of their reactivity with forage proteins after the plant has been chewed

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(Min *et al.*, 2003).The CT-protein interactions are most frequently based on hydrophobic and hydrogen bonding (Haslam, 1989). It is generally accepted that the interaction between protein and CT is pH dependent, each protein has a distinctive pH optimum (4.1 for bovine serum albumin (BSA) and 6.1 for fraction-10 leaf proteins (Jones and Mangan, 1977). Jones and Mangan, (1977) reported that CT can bind with protein at near neutral pH (pH 3.5-7.5) to form CT-protein complexes, which dissociate and release protein at pH less than 3.5. In most cases, CT are present in the leaves, seeds and stems of plants whilst in some forages, such as white clover and red clover, CT occur only in the flower petals (Barry, 1985).

2.5.7 Oxalic Acid

Some herbage species such as rhubarb, spinach and amaranthus specie contain high levels of oxalic acid. Oxalic acid, like phytic acid, has the ability to bind some divalent metals such as calcium and magnesium thereby interfering with their metabolism. According to Blood and Radostits, (1989), the ingestion of an excessive amount of oxalate could cause gastro-intestinal irritation, blockage of the renal tubules by calcium oxalate crystals, development of urinary calculi, hypocalcaemia, muscular weakness or paralysis. Plants generally tend to accumulate high oxalate levels during the early stages of growth. Xerophytic plants often tend to have high concentrations of oxalates.

Oxalic acid and its salts are widely distributed throughout the plant kingdom. Oxalate is simply a waste product in some plants, but in others, it may function in structural support, calcium storage and elimination, maintainance of ionic balance, or protection against predators (Hodgkinson, 1977). In most plants that produce oxalate in other than trace amounts, much of the calcium is present as calcium oxalate crystals. These crystals are held within cell vacuoles (Arnott and Pautard, 1970) and their formation is related to the calcium supply. This might be a strategy aimed at keeping the concentration of ionic calcium low, since high levels of ionic calcium are deleterious to growth.

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2.5.8 The Saponins

Saponins are secondary metabolites containing carbohydrate moieties attached to an aglycone which may either be steroidal or triterpenoid in structure. They are found in many plant species including those used for human food and livestock feeding. Available literature suggests that the dietary presence of saponins can either be beneficial or deleterious. The nutritional significance of saponins stem largely from their hypocholesterolaemic action, leading to the belief that they may prove useful in the control of human cardiovascular disease (Oakenful and Sidhu, 1983). The hypocholesterolaemic activity of dietary saponins may be due to the formation of some complexes with dietary cholesterol or their bile salt precursors which can then be made unavailable for absorption. Studies with dietary saponins (Johnson *et al.*, 1986) have shown that, besides lowering serum cholesterol, they also readily increase the permeability of the mucosal cells of the small intestine, thereby facilitating the uptake of materials to which the gut would not normally be permeable. Most saponins are highly surface-active and their ability to form addition complexes with sterols, including those associated with plasma membrane usually lead to membrane destabilization and cell lysis. The haemolytic property of many saponins *in vitro* has remained a charasteristic property frequently used as a basis for their quantitative assay. While there are suggestions that the consumption of saponins should be encouraged because of their hypocholesterolaemic activity, forage saponins have been reported by Cheeke *et al.*, (1978) to cause toxic and anorexic effects in the rat and swine, thereby limiting the feeding value of high-saponin animal feeds such as alfalfa.

2.5.9 The Alkaloids

The alkaloids constitute a diverse class of allelochemicals, some of which have great dietary importance. There have been reports, including those of Mulvihill (1972), that if ingested by ewes teratogenic alkaloids can alter normal foetal development resulting in foetal malformation. Forage alkaloids were implicated for the near epidemic increase in the birth of monstrous lambs with single mid-facial eyes in the Rocky Mountain region of the United States in the 1950s. Epidemiological studies showed that the embryos of ewes grazing ranges with extensive growth of veratrum (*Veratrum*

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californicum, Durand) were most susceptible. Feeding trials with purified *V. californicum* extracts clearly implicated cyclopamine and related alkaloids as the causal principles.

Quite apart from forage alkaloids, the need to develop rapid techniques to control the quality and evaluate the safety of alkaloid-containing foods has been stressed. The glycoalkaloids, solanine and chaconine present in potato and *Solanum spp* (Saito *et al.*, 1990; Aletor, 1991a) are heamolytically active and toxic to fungi and humans. Some of the toxicological manifestations of potato glycoalkaloids involve gastrointestinal upsets and neurological disorders, especially in doses in excess of 20mg/100g sample.

The toxic alkaloids are of importance as hazards to farm animals and to man. Among grazing animals, those which have access to plentiful pasture seem to learn not to eat the toxic plants. Ragworth *(Senecio jacoboca)* is frequently seen in many English meadows, but ragworth poisoning is not recognized as a major Veterinary problem in England. When pasture is scanty, however, the plants are eaten and the consequent loss of stock presents a serious economic problem in Australia. When contaminated hay or grain is fed, the animals cannot protect themselves by selecting what they eat (McLean, 1970). Poisoning in man has occurred by contamination of cereals and by the use of poisonous plants in traditional medicines. In the West Indies, it is an impartant cause of cirrhosis of the liver in man.

2.6 ALKALOID STUDIES IN THE WHOLE ANIMAL: VETERINARY STUDIES

The Veterinary studies have been reviewed by Sippel (1964) and by Bull *et al.* (1968). Different species present different syndromes. Cattle were among the earliest to be investigated, when the farmers of Nova Scotia noted that their cows fell ill after eating the imported weed, Stinking Willie (*Senecio jacoboca*). Other experiments with Senecio on cattle were reported by (Markson, 1960 and Thorpe and Ford, 1968), with Heliotropium by Bull *et al.*, (1951) and Kinnaird *et al.* (1968) with *Amsinckia* by Fowler (1968), and with *Crotolaria* by Bras *et al.* (1957). Cattle succumb rapidly within about a month, the common signs are violent diarrhea with tenesmus, wasting, a straddled gait, inability to stand, and death. Cattle fed *Crotolaria* develop ascites and veno-occlussive disease.

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Sheep are resistant to Senecio (Bull *et al.*, 1968). Field and experimental studies with Heliotropium and its alkaloids have been carried out by Bull *et al.* (1956) and Jago *et al.* (1969). Sheep are affected only by heliotrope and most survive one season's grazing. During the second season there are three pattern of illness, all different from that in cattle. Some die within a few days of transfer to lush pastures. From evidence collected among horses and rats this death is thought to be due to a high level of blood ammonia arising from the inability of the damaged liver to deal with a high protein intake Bull *et al.* (1956). The second group die less rapidly with haemoglobinuria and very high liver copper levels. The third group die much later, at least 9 months after the second grazing of *Heliotropium* (which is a seasonal plant), of gradual liver failure. These animals have small livers containing very large parenchymal cells. Sheep fed Crotolaria exhibit yet another syndrome. They die within three weeks with fluid in the thoracic cavity (Laws, 1968). The signs in horses are predominantly neurological, though stomach and oesophageal lesions are also reported. Rose *et al.* (1967a and 1967b) and Gardiner *et al.* (1965) investigated "walk-about" disease of horses and found it distributed over the same areas as *Crotolaria retusa*.The symptoms are compulsive walking in a straight line and butting the head against any obstruction encountered. Feeding with *Crotolaria retusa* produced the illness. The onset of neurological symptoms coincided with a steep rise of blood ammonia. Severe pathological changes in the liver were recorded at death.

Chickens and turkeys poisoned with *Crotolaria spectabilis* seed died with lesions in the liver, lung and muscle (Allen, 1963; Allen *et al.*, 1960; Allen *et al.*, 1963). Administration of senecio alkaloids to chickens can produce carcinomas (Campbell, 1956). This is the only instance of carcinogenesis due to these alkaloids outside the species commonly used in laboratory investigation (McLean, 1970). *Senecio* (Harding *et al.*, 1964) and *Crotolaria* (Emmel *et al.*, 1935) poisoning in pigs produce liver, lung and kidney lesions. Heliotrope poisoning in pigs (Burkser, 1948) produce liver lesions and gastric ulcerations. In dogs, heliotrope alkaloids give rise to liver failure in which ascites is the most prominent feature (Khanim, 1956).

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In summary, one cannot say that the Veterinary syndromes fall into a recognizable pattern. Acute liver damage is seen in poultry, chronic liver damage is seen in sheep, horse, pigs and dogs. Lung damage is seen in sheep, poultry and pigs. In addition, horses suffer neurological disturbances which may be secondary to chronic liver damage but a primary neurotoxic action of the alkaloids has not been ruled out. Sheep exhibit a haemolytic syndrome and cattle suffer an unexplained but fatal gastrointestinal disorder (McLean, 1970).

The differences may depend, not only upon the specie of animals affected but also on the plant consumed. The alkaloids of Crotolaria are noteably more likely to produce lung lesions, and less likely to produce tumours in laboratory animals. In field experiments, there is little control over the dose administered. In laboratory animals, a fairly simple picture of toxicity of the alkaloids emerges. Large doses cause an acute necrotic and vascular lesions in the liver. Smaller doses cause a progressive lesion in the liver, characterized by megalocytosis and a progressive lesion in the lungs. There is disputed carcinogenic effect in the liver (McLean, 1970). In Veterinary practice, the acute and chronic liver and lung lesions are frequently seen. Carcinogenesis is not reported. Other syndromes are common causes of death, viz diarrhea in cattle, neurological signs in horses and a haemolytic syndrome in sheep.

Young animals are much more susceptible to liver megalocytosis than adults. The view that young animals are particularly susceptible to other effects of the alkaloids is not supported by the published evidence. All the reported instances of human intoxication fall into the category of acute liver damage and its sequelae. There is no evidence on the often-discussed role of the alkaloids as liver carcinogen in man.

2.7. EFFECTS OF PLANT TOXINS ON ANIMALS

Toxic plants may grow together with forage plants and are therefore readily accessible to grazing animals. Poisonous plants are often refused by animals (many have repulsive smell or contain highly irritant juice) e.g. Euphorbia spp and are eaten only when other herbage is scarce, some highly poisonous plants are reported to be highly palatable (Van [illegible] et al., 1972). It is however an unfortunate fact that many animals develop a taste for poisonous plants. Clarke and Clarke (1975) reported that there were

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many instances on record of animals having to be forcibly restrained from returning to patches of poisonous plants after having recovered from poisoning by them. Plant poisoning in livestock could arise from the following:

i) Overgrazing of pastures and ranges especially during periods of moisture deficiencies that reduce forage production (Ewar and Hall, 1978).

ii) Animals that have trekked long distances or just arriving from shipping without sufficient feed and then turned on ranges may be poisoned. This is because hungry animals feed greedily on whatever is available, including poisonous plants, instead of selecting at a normal grazing rate. Animals forced to go on long distances without water may also eat any plant after gaining access to water and so poisoning often results (Clarke and Clarke, 1975).

iii) When poisonous plants are harvested with hay or their seeds become mixed with grain, it becomes difficult for the animals to separate the toxic elements from the good feed and poisoning frequently results. (Radcleff, 1970; Clarke and Clarke, 1975).

iv) Animals seem to have some sort of ability to learn to live with toxic plants, but when animals are newly introduced to a strange environment, they often show signs of poisoning during their first month, if toxic plants are present (Kingsbury, 1964). During periods of drought, animals will be compelled by hunger to browse any available plant (Abatan, 1992).

v) Occasionally, the poisoning may not be due to a plant but some fungi growing on it. An example is *Aspergillus flavus*, which causes aflatoxicosis, and *Claviceps purpurea*, which causes ergotism. This they do by attaching themselves to grasses and then render them toxic (Abatan, 1992).

vi) Other conditions that may predispose livestock to plant poisoning include:

a. Pica due to several causes like illness, central nervous system disorders etc. (Blood and Radostits, 1989).

b. Curious excited animals are likely to sample the plants they would otherwise not eat.

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c. Young animals are less discerning and are more susceptible to poisoning by toxic plants.

d. Plants that are different in texture e.g. sprayed weeds, lopped foliage often appear to be attractive.

2.8 Determination of Protein Quality of Foods

Many methods, approaches and techniques have been employed to accurately measure the nutritional quality of proteins (Miller and Bender, 1955; Miller, 1963; Mitchel and Grundel, 1986; Finke *et al.*, 1989). It appears that no single method is completely satisfactory. These methods can be classified broadly into two, namely chemical method and biological assay.

2.8.1 Chemical Method

This method determines the accurate amino acid profile of the protein. This involves three separate amino acid analysis viz. a standard amino acid analysis, one for tryptophan and one for sulphur amino acid. The amino acid score is calculated as mg of amino acid in 1 g of test protein divided by mg of amino acid in 1g of a reference protein. The reference protein is a protein of high biological value like lactalbumin or casein, which contains a specified pattern of amino acid. The limiting amino acid in the protein is an essential amino acid that shows the greatest difference in concentration from the same amino acid in a reference protein. The limitation of the chemical method is that it does not take into account the digestibility of the test protein and the internal absorption of the amino acid of the protein, but the advantage is that it requires less time to determine.

2.8.2 Biological Method

This involves the use of micro organisms, small animals and even human beings to evaluate protein quality. The microbiological methods are rapid, simple to operate and low in cost (Frank *et al.*, 1975). Protein quality has also being estimated using humans (Young and Scrimshaw, 1977). The limitation of this method is the prohibitive cost involved, long time required for the experiment and the need to obtain consent from participants and constituted authority. The most popular biological assay is that in which

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laboratory animals like mice, rats, chicken etc are used. The protein quality is determined either by growth experiments or by nitrogen balance methods.

2.8.3 Growth Experiments

2.8.3.1 Protein Efficiency Ratio (PER)

This is the most common method used for animal assay to determine protein quality using the growth experiment. This method is very sensitive in assessing damage to proteins due to processing and ranking food according to their protein quality (Solberg *et al.*, 1979). Basically, weanling rats or mice are fed with purified diets for a long period of days. The purified diet is prepared and expected to be nutritionally adequate to promote expected growth rates in laboratory animals (NAS, 1972). A reference protein containing 10% protein from reference proteins are also included. 5 to 10 rats are assigned to each diet. The animals are housed individually in stainless wire-bottom cages in well-ventilated rooms. After an acclimatization period of 3-5 days, the animals are fed on the experimental diets for 28 days (Campbell, 1963). During this period, food and water are given *ad-libitum* and feed intake and weight gain recorded at intervals. Using total feed intake and final body weight, the net protein intake and body weight increase are calculated. The PER is calculated as body weight gain per net protein intake.

$$PER = \frac{\text{weight gain (g/rat/day)}}{\text{Protein intake (g/rat/day)}}$$

Generally, a PER below 1.5 approximately describes a protein of low quality, between 1.5 and 2.0, an intermediate quality and above 2.0 good to high quality. In spite of its simplicity, the PER has many limitations. It does not make allowance for maintenance requirements of the test animal. The result of PER varies with food consumption. The assumption that the gain in body weight is indicative of the protein tissue laid down is not always valid (Bender and Doell, 1957). The cost and time required for the experiment are high and the method may be unreliable for some low quality protein foods (Fried, 1977). In order to overcome some of these limitations, some modifications have been added. A metabolic group, fed on a protein-free diet has

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been introduced to create allowance requirements for animals placed on the test protein diets.

2.8.3.2 Net Protein Ratio (NPR)

NPR method is considered to be an improvement over the PER method for measuring the protein quality of foods (Sarwar and McDonough, 1990). NPR is calculated as weight gain of test animal plus weight loss of animals fed on non-protein basal diet, divided by weight of protein consumed.

$$NPR = \frac{\text{weight of test group + mean weight loss on N-free group}}{\text{weight of protein intake}}$$

2.8.3.3 Protein Retention Efficiency (PRE)

This is a measure of protein quality, comparing its protein efficiency ratio with the weight loss of a test group fed no protein. It is a product of NPR and 16 (Bender and Doell, 1957).

Some authors (Bender and Doell, 1957; Khan and Ghafoor, 1978, Laufer and Lajolo, 1990) have reduced the 28-day PER method to shorter assay periods ranging from 10-21 days. Shorter assays of PER have been reported to give low PER values. The long trials allow the animals to become habituated to the diet, particularly in cases where the food has low palatability. The acceptability and consumption of the diet is a critical factor in methods based on weight gain measurements. Fluctuations in the quality of food consumed may increase the variability of PER values (Subba-Rau *et al.*, 1972). Variations in PER values even for the same food had been reported (Campbell, 1963). The main reason for this variation is the short period of the experiment.

2.8.3.4 Nitrogen Balance Methods

This is a biological assay method in which the efficiency with which dietary protein is converted to body protein in laboratory animals is measured. The net protein utilization (NPU) reflects the portion of protein intake, which is retained for maintenance and increase in body proteins. Two factors namely, the digestibility of the protein and the biological value of the protein will determine the NPU value. The biological value is directly related to biological metabolism of the protein and therefore the rate of utilization of the proteins absorbed by the body (g).

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Net Protein Utilization (NPU): According to (Philips *et al.*, 1981).

$$NPU = \frac{I - (F-M) - (U-E)}{I} \times 100 \quad OR \quad \frac{BV \times TD}{100}$$

Where I = nitrogen intake (mg)

F = nitrogen excreted in faeces (mg)

M = metabolic faecal nitrogen (from basal diet) (mg)

U = nitrogen excreted in urine (mg)

E = endogenous urinary nitrogen from basal diet (mg).

BV = biological value

Biological Value (BV): It is defined as the fraction of the absorbed nitrogen that is retained in the body for maintenance and growth (Philips *et al.*, 1981).

$$BV = \frac{I - (F-M) - (U-E)}{I-(F-M)} \quad OR \quad \frac{NPU \times 100}{TD}$$

2.8.3.5 Digestibility of Proteins

The best method to determine the digestibility of a protein is by *in vivo* animal feeding studies. The apparent digestibility is given by the following equation:

$$\text{Apparent digestibility} = \frac{[N \text{ consumed} - \text{faecal } N]}{N \text{ consumed}} \times 100$$

The true digestibility is obtained by making allowance for endogenous faecal loss by animals fed on the protein-free diet.

$$\text{True digestibility} = \frac{[N \text{ consumed} - \text{faecal } N - \text{Metabolic } N]}{N \text{ consumed}} \times 100$$

However, *in vitro* techniques have also been developed and proven to be useful and reliable in estimating true digestibility. The technique is based on the addition of proteolytic enzymes like trypsin, chymotrypsin and peptidases to the protein substrate maintained at optimum incubation conditions for the enzyme. The *in vitro* technique only gives an approximation of the true digestibility. Mitchel and Grundell (1986), found that protein digestibility tended to be underestimated by *in vitro* tests.

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2.9. Anti-oxidants

An antioxidant is defined as any substance, which when present at a low concentration compared with those of an oxidizable substrate, such as fats, proteins, carbohydrates or DNA, significantly delays or prevent the oxidation of the substrate (Halliwell and Gutteridge, 1990). Also classified as antioxidants are acidic compounds (including phenols) useable in foods, which can readily donate an electron or a hydrogen atom to a peroxyl or alkoxyl radical to terminate a lipid peroxidation chain reaction or to regenerate a phenolic compound which can effectively chelate a prooxidant transition metal. Antioxidant means "against oxidation". Antioxidants work to protect lipids from peroxidation by radicals. Antioxidants are effective because they are willing to give up their own electrons to free radicals. When a free radical gains the electron from an antioxidant, it no longer needs to attack the cell and the chain of oxidation reaction is broken (Davies, 1988).

A substance may exert antioxidant action by inhibiting the generation of reactive oxygen species, by scavenging free radicals, or by raising the levels of endogenous antioxidant defenses and regulating the expression of the genes coding for antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxides (Davies, 1988). There are two types of antioxidants in the human system; they are enzymatic (natural) antioxidants and non-enzymatic (synthetic) antioxidants. Enzymatic (natural) antioxidants neutralize reactive oxygen species and free radicals thereby preventing it from damaging the cellular structure. They are composed of the enzyme superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase which causes the reduction of hydrogen peroxide to water and alcohol.

(i) Superoxide dismutase (SOD)

Superoxide dismutase removes superoxide radical by greatly accelerating its conversion to hydrogen peroxide and molecular oxygen. In human cells, two forms of this enzyme exists; a cytosolic copper and zinc-containing enzyme, and a mitochondrial manganese-containing enzyme.

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$$O_2^- + O_2^- \xrightarrow[2H^+]{SOD} H_2O_2 + O_2.$$

Superoxide dismutase is present in high concentration in all tissues and it has a high catalytic efficiency, hence, it provides the cells with a high degree of cellular protection against superoxide anion under normal condition. It is localized in the nuclei, and in the seminiferous tubules of the testes (Ookawara *et al.*, 2002).

The activity of SOD in the cells and in the extracellular fluid is very important in the prevention of diseases closely associated with oxidative stress, for instance, cardiovascular diseases, Alzheimer's disease, Parkinson's disease and many others (Pollack and Leeuwenburgh, 1999). Oxidative damage develops when antioxidant potential is reduced and/or when factors contributing to oxidative stress increase (Ibrahim *et al.*, 2000; Poljicak-Milas *et al.*, 2004; Milinkovic-Tur *et al.*, 2007).

(ii) Glutathione Peroxidase (GSH-Px)

GSH-Px is the most important hydrogen peroxide-removing enzymes in the cell. This enzyme requires selenium (as selenium cysteine at the active site) for its action. GSH-Px catalyzes the reduction of H_2O_2 and organic hydroperoxides as follows:

$$2GSH + H_2O_2 \xrightarrow{GSH\text{-}Px} GSSG + 2H_2O$$

$$2GSH + ROOH \xrightarrow{GSH\text{-}Px} GSSG + H_2O + ROH$$

Glutathione is used as an electron donor in the reduction of reactive oxygen species.

(iii) Catalase

Catalase is a tetrameric enzyme containing four heme groups that allow the enzyme to react with hydrogen peroxide (Chelikani *et al.*, 2004; Zamocky and Koller, 1999). It completes the detoxification started by superoxide dismutase by converting H_2O_2 generated by SOD to water and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

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Catalase is present in the highest concentration in peroxisomes, cell organelles located close to the mitochondria in eukaryotic cells (del Rio *et al*., 1992) but it is also found in smaller concentrations in the mitochondria and the cytosol. The enzyme uses the bound Fe atoms of its heme groups in conjunction with asparagines, histidine and tyrosine residues at the active site, to carry out its decomposition of H_2O_2. Its mechanism of action is a ping-pong mechanism where its cofactor (Fe) is oxidized by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate (Hiner *et al*., 2002).

Catalase catalyzes hydrogen peroxide breakdown only at high concentrations of the substrate. At low concentrations, H_2O_2 appears to be metabolized by peroxides such as peroxiredoxins. High levels of catalase activity are found in the liver, kidney and red blood cells.

(iv) Glutathione

Glutathione, a thiol containing tripeptide (γ-glutamyl-cysteinyl-glycine) is present in virtually all living cells in high concentration. It is a co-substrate of glutathione peroxidase and transferase. However, glutathione readily reacts with free radicals like O_2^-, OH^-, RO and ROO. Glutathione can serve as both a nucleophile and an effective reductant, it plays an important role in a variety of detoxification processes. This includes the nullification of peroxide damage as evidenced by GSH depletion, which increases the susceptibility of animals against cytotoxicity and affects drug intervention in neoplastic disease (Al-Turk *et al*., 1987). Besides the antioxidant function of GSH, it also provides the reducing equivalent needed by glutathione peroxidase in its antioxidant function. Therefore, high concentrations of GSH have been observed in rat and mouse testes, reproductive trait fluids, and epididymal spermatozoa (Calvin *et al*., 1981). The liver is known to have one of the largest GSH concentrations.

(v) Vitamin C (Ascorbic Acid)

Ascorbic acid is a water soluble free radical scavenger of high biological relevance and successfully prevents detectable oxygen damage under all types of oxidative stress. The antioxidant properties of ascorbic acid involves its ability to donate an electron to a biological radical, with its concomitant oxidation to ascorbyl radical.

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Ascorbic acid is known to act as an antioxidant both in *in vivo* and *in vitro* studies. It plays an important role in detoxification of reactive intermediates produced by cytochrome P_{450}, which detoxify xenobiotics (Prakassam *et al.*, 2005).

When compared with other water soluble antioxidants, vitamin C offers the most effective protection against plasma lipid peroxidation (Jialal *et al.*, 1990). For example, Frei *et al.*, (1990) showed that ascorbate disappeared more rapidly than urate and α-tocopherol when peroxyl radicals were generated using water soluble radical agent 2, 2' azobis (2 amidimopropane hydrochloride) which generates peroxyl radicals. Vitamin C is widely distributed in mammalian tissues, but it is present in relatively high amount in the adrenal and pituitary glands; lesser amounts are found in the testes, liver, spleen, pancrease and brain. Ascorbic acid serves as both an antioxidant and a prooxidant. It can neutralize ROS such as H_2O_2 (Shigeoka *et al.*, 2002). However, under certain conditions, it may promote oxidation of other substances (Benedict *et al.*, 1984).

(vi) Vitamin E (Tocopherols and tocotrienols)

This is a complex mixture of structurally-related lipophilic compounds which all have antioxidant character (Herrero and Barbas, 2001; Packer *et al.*, 2001). The most potent scavenger of ROS is α-tocopherol which is commonly taken as vitamin E and it plays a vital role in protecting membranes from oxidative damage by trapping peroxy radicals in cellular membranes (Traber and Atkinson, 2007). In doing this, it is converted to α-tocopheroxyl radical but the active vitamin E is regenerated by vitamin C, glutathione and ubiquinol (Wang and Quinn, 1999). γ-tocopherol is also a potent scavenger of reactive nitrogen species like NO and peroxynitrite (Brigelius-Flohe and Traber, 1999). Both tocopherols and tocotrienols also reduce the risk of cardiovascular disease by preventing LDL oxidation.

(vii) β- Carotene

β- Carotene is the pro-vitamin A found in the membranes of certain tissues (e.g. the retina) in high concentrations. β- Carotene is another non-enzymatic antioxidant capable of inactivating singlet molecular oxygen. It is also capable of terminating peroxidative chain reaction based on its ability to react directly with free radicals.

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Other non-enzymatic antioxidants include vitamin-like such as coenzyme Q10 (CO Q10), glutathione, some common phytochemicals such as flavonoids, which have being reported to have antioxidant characters.

(viii) Selenium

Selenium (Se) is a constituent element of the entire defence system that protects the organism from harmful free radical action. Organic selenium is more thoroughly reabsorbed and more efficiently metabolised than its inorganic equivalent, which is poorly resorbed and acts more as a prooxidant, provoking glutathione oxidation and oxidative damage to the DNA (Levander, 1983; Schrauzer, 2000; Wycherly *et al.*, 2004). Resorbed selenomethionine is primarily incorporated into the proteins of the muscles, erythrocytes, of the pancrease, liver, stomach, kidney and the gastro-intestinal tract mucous membrane. Its metabolism is connected with protein metabolism in the body (Schrauzer, 2000), and is accessible for the synthesis of glutathione and selenoproteins through which it displays its physiological action.

The most important metabolic role of selenium is shown in the activities of the selenoenzymes glutathione peroxidase (GSH-Px) and thioredoxin reductase. The enzyme GSH-Px, together with superoxide dismutase (SOD) and with catalase (CAT), protects cells from (hydrogen or lipid) peroxidation. Glutathione peroxidase is mostly a cytosilic enzyme. In small quantities, it is found in mitochondrial membranes and in endoplasmic reticulum. Another important enzyme in the antioxidant system is SOD, whose presence in the cell allows a rapid dismutation of O_2^- into O_2 and H_2O_2. For the major part, Cu, Zn-SOD is found in the cytosol, and Mn-SOD in the mitochondria (Fridovich, 1997). Catalase, acting together with SOD, transforms H_2O_2 into H_2O and O_2 (Michelis *et al.*, 1994). Catalase activity, as well as the activity of other antioxidative enzymes, depends on the presence of antioxidants in the diet. Thus, the activity of GSH-Px in the blood of young chicks depends on the quantity of selenium (Kuricova *et al.*, 2003), and the activity of catalase in chicken erythrocytes depends on the quantity of copper and selenium in the diet (Bozcaya *et al.*, 2001).

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2.9.1 Free Radicals

Oxidation is a reaction involved in the metabolism of human body. However, under the impact of various factors, it could sometimes lead to the formation of an excessive amount of residual metabolism products, the so-called free radicals. The free radicals and other reactive oxygen compounds trigger chain reactions of oxidation within the human body,as a result, leading to the damage of cells and interruption of their functions (Bowen, 2003). A free radical (radical) is an atom or group of atoms that have one or more unpaired electrons. Radicals can either have positive, negative or neutral charge. They are formed as necessary intermediates in different normal biochemical reactions, but when generated in excess or not appropriately controlled, radicals can wreak havoc on a broad range of macromolecules. A well known feature of radicals is that they have extremely high chemical reactivity, which explains not only their normal biological activities, but how they inflict damage on cells (Bowen, 2003). Free radicals are molecules produced through normal digestive processes and they are involved in aging, as well as numerous chronic illnesses such as cancer and cardiovascular disease. Free radicals are produced inside (and also released towards the cytosol) organelles, such as the mitochondrion.

2.9.2 Reactive Oxygen Species

There are many types of radicals, but those of major concern to biological systems are derived from oxygen, and known collectively as reactive oxygen species. Oxygen has two unpaired electrons in separate orbitals in its outer shell. This electronic structure makes oxygen especially susceptible to radical formation. Sequential reduction of molecular oxygen (equivalent to sequential addition of electrons) leads to formation of a group of reactive oxygen species (Bowen, 2003).

Reactive oxygen species (ROS) include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are generally very small molecules and form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signalling. However, during times of environmental stress, ROS levels can increase dramatically, which can result in significant damage to cell structures. This

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leads to a situation known as oxidative stress. They are also generated by exogenous sources such as ionizing radiation (Sen, 2003). Reactive oxygen species are:

(i). Molecules like hydrogen peroxide/peroxide ion.

(ii). Radicals like the hydroxyl radical. It is the most reactive of them all.

(iii). The superoxide anion which is both ion and radical.

(iv). Nitric oxide (NO) etc.

The reduction of oxygen to water proceeds via one electron at a time. In the mitochondrial respiratory chain, complex IV (cytochrome oxidase) retains all partially reduced intermediates until full reduction is achieved. Other redox centres in the electron transport chain, may however leak electrons to oxygen, partially reducing this molecule to superoxide anion (O_2^-). Although O_2^- is not a strong oxidant, it is a precursor of most other reactive oxygen species, and it also becomes involved in the propagation of oxidative chain reactions. The mitochondrion appears to be the main intracellular source of these oxidants (Barja, 1999).

2.9.3 Formation of Reactive Oxygen Species

Oxygen-derived radicals are generated constantly as part of normal aerobic life. They are formed in mitochondria as oxygen is reduced along the electron transport chain. Reactive oxygen species are also formed as necessary intermediates in a variety of enzyme reactions. Examples of situations in which oxygen radicals are produced in cells include:

(i). The interaction of ionizing radiation with biological molecules

(ii). As an unavoidable byproduct of cellular respiration. Some electrons passing "down" the electron transport chain leak away from the main path (especially as they pass through ubiquinone) and go directly to reduce oxygen molecules to the superoxide anion.

(iii). Synthesized by dedicated enzymes in phagocytic cells like neutrophils and macrophages, *NADPH oxidase (in both type of phagocytes) and *Myeloperoxidase (in neutrophils only).

(iv). Several xenobiotics interact with the mitochondrial electron transport chain, increasing the rate of oxygen radicals (O_2^-) production through two different

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mechanisms. Some of these compounds stimulate oxidative stress because they block electron transport, increasing the reduction level of carriers located upstream of the inhibition site. Other xenobiotics may accept an electron from a respiratory carrier and transfer it to molecular oxygen, stimulating $O_2^{\cdot-}$ formation without inhibiting the respiratory chain (Barja, 1999).

"Oxidative stress" (OS) is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as ROS (Sikka *et al.*, 1995). Oxidative stress (OS) precipitates the range of pathologies that currently are thought to afflict the reproductive function (Joyce, 1987; Sharma and Agarwal, 1996; Maneesh *et al.*, 2005a). OS down regulates the steroidogenic activity leading to altered testicular function (Maneesh *et al.*, 2005a). The generation of ROS has become a real concern because of their potential toxic effects at high levels on sperm quality and function (Sikka, 1996).

Male germ cells at various stages of differentiation from pachytene spermatocytes to mature caudal epididymal spermatozoa have the potential to generate ROS (Agarwal *et al.*, 1992; Agarwal *et al.*, 1994a). Spermatozoa may generate ROS in two ways: (i). the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the level of the sperm membrane (Shekarriz *et al.*, 1995) and in (ii). The NADPH-dependent oxido-reductase (diphorase) at the level of mitochondria (Lopes *et al.*, 1998).

2.9.4 Nitric Oxide

Nitric oxide is widely known for its very important role in the mediation of signal transduction via activation of guanylase cyclase and is also involved in the regulation of tissue viability. Nitric oxide has also been discovered to play a role in enhancing malaria resistance and is also involved in coronary heart disease, acute inflammation and some other medical conditions. Nitric oxide is a vasodilator resulting from the breakdown of arginine to citrulline, in a reaction catalysed by a family of NADPH-dependent enzymes called nitric oxide synthases. It has recently been reported that the mitochondrial matrix contains a unique form of nitric oxide synthase (Ghafourifar and Richter, 1997; Giulivi *et al.*, 1998; Alvarez *et al.*, 2003). Although its

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physiological role is still unclear, the formation of nitric oxide in mitochondria may have important consequences because this compound binds to haem groups from cytochromes (in particular cytochrome oxidase) and inhibits respiration (Poderoso *et al.*, 1996). This may in turn, stimulate $O_2^{\bullet-}$ formation (for example from Complex I) which in turn may react with more nitric oxide forming peroxynitrite, an oxidant capable of inhibiting important enzymes and affecting mitochondrial integrity (Cassina and Radi, 1996; Radi *et al.*, 2002b).

2.9.5 Peroxyl Radicals

They are formed during lipid peroxidation reactions such as the oxidation of polyunsaturated fats leading to the deterioration of lipid-containing foods. Lipid peroxidation may be initiated by any species that has sufficient reactivity to remove a hydrogen atom from a polyunsaturated fatty acid, e.g. arachidonic acid and linoleic acid. For example, arachidonic acid is known to contain a number of methylene-interrupted double bonds which are particularly prone to hydrogen atom removal.

The peroxy radicals have marked effects on vascular function because of their ability to antagonize the actions of some of the stereospecific products formed by cyclooxygenase and lipoxygenase enzymes. Under aerobic conditions, conjugated dienes are able to combine with O_2 to give a peroxyl (or peroxy) radical, ROO. The formation of peroxy radicals *in vivo* appears to be enhanced under conditions of oxidative stress such as smoking, exposure to xenobiotics and pathological conditions associated with inflammation.

2.9.6 Superoxide Radicals

Superoxide is oxygen centered radical with selective reactivity. The production of superoxide by the mitochondrial respiratory chain occurs continuously during normal aerobic metabolism. It is estimated that 1-2% of all the electrons traveling down the mitochondrial respiratory chain never makes it to the end, but instead form superoxide. In addition to the mitochondrial respiratory chain, there are other endogenous sources of superoxide production. In particular, when leukocytes (white blood cells) encounter microorganisms or other pathogens invading our bodies, they start to generate large amounts of superoxide. Superoxide formation occurs on the outer mitochondrial

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membrane, in the matrix and on both sides of the inner mitochondrial membrane (Hans *et al.*, 2003).

2.9.7 Hydrogen Peroxide

Hydrogen peroxide is not a free radical but a compound and of great importance in that it gradually oxidizes a number of endogenous compounds. It is formed in the body by the action of superoxide dismutase on oxygen radical and also by the action of other oxidases. Hydrogen peroxide is only highly active in high concentrations and not in low concentrations. It attacks very important metabolic processes such as in glycolysis where it inactivates glyceraldehydes-3-phosphate dehydrogenase. Hydrogen peroxide (H_2O_2) in turn may be fully reduced to water or partially reduced to hydroxyl radical (OH), one of the strongest oxidants in nature. The formation of OH is catalysed by reduced transition metals, which in turn may be re-reduced by O_2^- propagating this process (Liochev and Fridovich, 1999). Hydrogen peroxide is mostly decomposed by the enzyme glutathione peroxidase. In the liver, mitochondria account for about one third of the total glutathione peroxidase activity (Chance *et al.*, 1979).

2.9.8 Hydroxyl Radicals

The hydroxyl radicals are a group of highly reactive oxygen radicals. The typical hydroxyl radical results in the formation of another radical species, after reacting with different substrates and the resulting radical species usually has a lower reactivity than that of the hydroxyl radical. Hydroxyl radicals react with almost all macromolecules (proteins, fatty acids, DNA etc) or biological molecules in its vicinity. Hydroxyl radicals are derived from the decomposition of hydrogen peroxide in the presence of oxidized iron (Fe^{2+}) via the famous "Fenton reaction".

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$ (Fenton reaction).

Secondly, HO is formed by the interaction of superoxide with hydrogen peroxide through the Haber-Weiss reaction (Haber and Weiss, 1934).

$O_2 + H_2O_2 \rightarrow O_2 + H_2O + HO$ (Haber-Weiss reaction).

2.9.9 Activities of the Reactive Oxygen Species

Reactive oxygen species (ROS) should not be regarded as generally bad as they are also generated in a number of reactions essential to life. Strong oxidants like the

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various ROS can damage other molecules and the cell structures of which they are a part. Reactive oxygen species are implicated in cellular activity to a variety of inflammatory responses including cardiovascular disease. They may also be involved in hearing impairment via cochlear damage induced by elevated sound levels, ototoxicity of drugs such as cisplatin, and in congenital deafness in both animals and humans. Redox signaling is also implicated in mediation of apoptosis or programmed cell death and ischaemic injury. Specific examples include stroke and heart attack (Sen, 2003).

Generally, the most common harmful effects of reactive oxygen species on the cell are DNA damage, oxidations of polyunsaturated fatty acids in lipids (as in lipid peroxidation), oxidations of amino acids in proteins and oxidative inactivation of specific enzymes by oxidation of cofactors (Sen, 2003).

2.9.9.1 Testicular Antioxidant Defences

The relative lack of cytoplasmic space is a striking feature of human spermatozoa and may contribute to their vulnerability to oxidative stress, by reducing their capacity for antioxidant defense. In contrast, seminal plasma is a potent source of antioxidants, including SOD (Kobayash *et al.*, 1991), uric acid, α tocopherol and vitamin E (Jones *et al.*, 1979; Zini *et al.*, 1993). In addition, spermatozoa are known to be coated in lactoferrin, an iron-binding protein that plays a significant role in removing this important transition metal from sites where it may catalyse peroxidative damage. Seminal plasma is not the only extracellular fluid in male reproductive tract to possess antioxidant factors. Reports of unique secreted forms of glutathione peroxidase and SOD in mammalian epididymis emphasize the importance of epididymal plasma in protecting spermatozoa from peroxidative damage during their prolonged storage in caudal epididymis.

It is thus clear that ejaculated spermatozoa may be subject to oxidative stress, and there are intracellular and extracellular mechanisms to protect these cells from damage after spermatogenesis, during epididymal transit, and after ejaculation. However, oxidative stress may also arise during spermatogenesis, and may casually be involved in the pathophysiology of male infertility. The antioxidant enzymes catalase, SOD, GPx and glutathione transferase (GTR) and hexose monophosphate shunt (HMS)

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are present in rat testis and significant changes in these enzymes are observed during testicular development. The ratio SOD: Catalase plus GPx in the testis is high, possibly contributing to the vulnerability of the testis to oxidative stress. A consequence of a dependence of the testis on SOD as an antioxidant defense strategy is the vulnerability of testicular SOD to thermal inactivation (Aitotupa and Huhtaniemi, 1992).

2.9.9.2 Sources of Reactive Oxygen Species (ROS) in Semen

There is a controversy as to whether the source of ROS in semen of subfertile men originates in the spermatozoa themselves, or in the infiltrating leucocytes (Aitken and West, 1984; Aitken and Clarkson, 1987). They trigger their defensive role directly by ROS synthesis or indirectly by other neighbouring white cells via soluble factors such as cytokines. Spermatozoa usually eliminate excess cytoplasm, and the presence of retained cytoplasmic droplets has been associated with reduced fertility. This is because there may be a link between ROS generation and residual cytoplasm in human spermatozoa through cytoplasmic glucose-6-phosphate dehydrogenase (G6PDH) producing nicotininamide adenine dinucleotide phosphate (NADPH) that in turn serves as a source of ROS (Cummins *et al.*, 1994).

2.9.9.3 Spermatozoa and Lipid Peroxidation (LPO)

Lipid peroxidation is a free radical-mediated process leading to oxidative deterioration of polyunsaturated lipids. Under normal physiological conditions, low concentrations of lipid peroxide are found in plasma and tissues (Prakasam *et al.*, 2005).

Human spermatozoa are known to be susceptible to loss of motility in the presence of exogenous H_2O_2, as a consequence of lipid peroxidation. This susceptibility of human spermatozoa to oxidative stress is a consequence of the abundance of unsaturated fatty acids in the sperm plasma membrane, the presence of which gives this structure the fluidity it needs to engage in the membrane fusion events associated with fertilization. Peroxidation of polyunsaturated fatty acids (PUFAs) in sperm cell membranes is an autocatalytic, self propagating reaction, which gives rise to cell dysfunction associated with loss of membrane function and integrity. Lipid peroxidation is one of the cellular pathways involved in oxidative damage. Oxidative stress, which is an excessive production of reactive oxygen species, can damage cells by triggering lipid

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peroxidation and by altering protein and nucleic acid structures (Sherm *et al.*, 1994; Pinkus *et al.*, 1997).

2.9.9.4 Reactive Oxygen Species and Sperm Physiology

Until recently, ROS were exclusively considered toxic to the human spermatozoa, although a strong body of evidence suggests that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities (Kovalski *et al.*, 1992; Saleh *et al.*, 2002; Jannsen *et al.*,1993; Spitteler, 1993). Theoretically, cellular damage in the semen is the result of an improper balance between ROS generation and scavenging activities i.e. positive oxidative stress status (OSS), a situation in which there is a shift towards prooxidants, because of either excesss ROS or diminished antioxidants. Levels of antioxidants in seminal plasma from infertile men are significantly low (Halliwell, 1990). However, pathological levels of ROS detected in semen from infertile men are more likely a result of increased ROS production rather than reduced antioxidant capacity of the seminal plasma (Aitken and Fisher, 1994).

With respect to sperm function, experimental evidence is emerging that ROS are involved in several fundamental mechanisms of sperm physiology. The precise mechanisms involved are presently being investigated, but there is evidence that the capacity for generation of ROS and antioxidative mechanisms change during epididymal transit and are part of the regulatory process (Fisher and Aitken, 1997; Tramer *et al.*, 1998). The available evidence indicates that the spermatozoa of fertile men produce extremely low levels of ROS, and yet, paradoxically, these molecules appear to be important mediators of normal sperm function (Aitken and Clarkson, 1987; de Lamirande and Gagnon, 1993). Superoxide anion, for example, has been shown to stimulate the hyperactivation of human spermatozoa via pathways that are susceptible to inhibition by superoxide dismutase. Evidence has also been obtained to suggest a role for ROS in the induction of the acrosome reaction in the hamster spermatozoa (Bize *et al.*, 1991). In this case, the active molecule appears to be H_2O_2, rather than O_2^-, since the induction of the acrosome reaction could be blocked by the inclusion of catalyst in the incubation medium. This suggests that the generation and release of H_2O_2 by these cells is an important element in their activation. Physiologically, the mechanism of action of

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ROS in normal human spermatozoa may be due to the ability of H_2O_2 to enhance tyrosine phosphorylation through the inhibition of tyrosine phosphatase activity (Hecht and Zick, 1992). There is good evidence to suggest that the activation of mammalian spermatozoa on contact with the zonal pellucida is mediated by a tyrosine kinase-type receptor that autophosphorylates when oligomerized by the zona glycoprotein.

2.9.9.5 Biological Implications of Lipid Peroxidation and Oxidative Stress to Spermatozoa

Spermatozoa, unlike other cells, are unique in structure, function and susceptibility to damage by lipid peroxidation (Alvarez *et al*., 1987). In general, the most significant effect of lipid peroxidation in all cell is the perturbation of membrane (cellular and organellar) structure and function (transport processes, maintenance of ion and metabolite gradients receptor-mediated signal transduction, etc), low levels of NADPH and glutathione, as a result of the increased activity of glutathione peroxidase to remove metabolites of lipid peroxidation, will further affect cellular calcium homeostasis (Sikka, 1996). Besides membrane effects, lipid peroxidation can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to MDA resulting in strand breaks and cross linking (Ernster, 1993). ROS can also induce oxidation critical SH groups in proteins and DNA, which will alter structure and function of spermatozoa with an increased susceptibility to attack by macrophages (Aitken *et al*., 1989). Reduced glutathione (GSH) is known to protect the cellular system against the toxic effects of lipid peroxidation (Garg and Bansal., 1996).

2.9.9.6 Impairment of Sperm Motility

The increased formation of ROS has been correlated with a reduction of sperm motility (Lenzi *et al*., 1993; Armstrong *et al*., 1999). The link between ROS and reduced motility may be due to a cascade of events that results in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm oocyte fusion (deLamarinde and Gagnon, 1995). High levels of ROS disrupt the inner and outer mitochondrial membranes resulting in release of cytochrome C protein from the mitochondria that

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activates the caspases and induces apoptosis (Sentman *et al.*, 1991; Maneesh *et al.*, 2005b). LPO is one of the cellular pathways involved in oxidative damage. Oxidative stress, which is an excessive production of reactive oxygen species, can damage cells by triggering lipid peroxidation and by altering protein and nucleic acid structures (Shem *et al.*, 1994; Pinkus *et al.*, 1997).

Another hypothesis is that H_2O_2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as Glucose-6-Phosphate Dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the Hexose Monophosphate Shunt, which in turn controls the intracellular availability of NADPH. This, in turn, is used as a source of electrons to fuel the generation of ROS by an enzyme system known as NADPH oxidase (Aitken, 1997). Inhibition of G6PD leads to decrease in availability of NADPH and a concomitant accumulation of oxidized glutathione and reduced glutathione. This can reduce the antioxidant defences of the spermatozoa and peroxidation of membrane phospholipids (Griveau *et al.*, 1995).

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CHAPTER THREE

MATERIALS AND METHODS

3.1 EXPERIMENT ONE

3.1.1 Sources and Description of *Lablab purpureus*

Sources: The *Lablab purpureus* seeds used for these studies were obtained from the International Institute for Tropical Agriculture (I.I.T.A) in Ibadan, Oyo State. The identification of the varieties was also done by an Agronomist, Dr. Linus Frank, at the Rice, Cereals and Maize Development (RCMO) unit of I.I.T.A, Ibadan.

3.1.2 General appearance of *Lablab purpureus* seeds

The general appearance of *L. purpureus* seeds is shown in Table 3.

Table 3 General appearance of *Lablab purpureus* seeds

Variety name	Rongai white	Rongai brown	Highworth black
Accession Number	NAPRI 4	P₁509 114	Grif 12293
Seed colour	White	Dark brown	Black
Seed coat texture	Hard corneous	Hard corneous	Hard corneous
General description	Oval/oblong	Oval/oblong	Oval/oblong
Seed weight (g/100 seeds)	23	26	23

3.1.3 Determination of the Proximate Composition of *Lablab purpureus* seeds varieties

METHODOLOGY

3.1.3.1 Crude Protein Determination

This was done according to AOAC (1999). The crude protein content was determined by the Kjeldahl method and three steps of analysis was followed, viz: digestion, distillation and titration. Into two semi-micro Kjeldahl tubes were weighed 0.5g of finely grounded dried sample (*Lablab purpureus* seeds) in duplicates, ensuring that all the sample materials got to the bottom of the tubes. To these were added one

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tablet each of selenium as catalyst and 10ml of concentrated H_2SO_4. Then, the two semi-Kjeldahl tubes and its contents were set in the appropriate holes of the digestion block heater in an inclined position.

The digest was cooled and carefully transferred into 100ml standard flask; the Kjeldahl tube was thoroughly rinsed with distilled water and the standard flask was made up to mark with the distilled water and shaken. Then from the 100ml standard flask, 5ml portion of the digest were pipetted into the body of the apparatus via, the small funnel appeture. To this was added 5ml of 40% (w/v) NaOH through the same opening with the 5ml pipette. The mixture was steam-distilled for 2 minutes into a 50ml conical flask containing 10ml of 2% Boric acid solution mixed with indicator (i.e. mixture of 0.016% methyl-red and 0.083% Bromocresol green in alcohol) and placed at the receiving tip of the condenser. The boric acid plus indicator solution changes colour from pinkish-red to green, showing that all the ammonia liberated have been trapped.

The green colour solution obtained was then titrated against 0.01M HCl contained in a 50ml burette. At the end point, the green colour turns to wine colour which indicate that all the Nitrogen trapped as Ammonium Borate $((NH_4)_2BO_3)$ have been removed as Ammonium Chloride (NH_4Cl).

Without the addition of 0.5g of the *Lablab purpureus* seeds, the process of digestion, distillation and titration was carried out as follows:

$$\% \text{Nitrogen} = \frac{(\text{Titre value} - \text{Blank titre}) \times AN \times MA \times DF \times 100}{\text{Weight of sample (mg)}}$$

where AN = Atomic mass of Nitrogen

MA = Molarity of Acid

DF = Dilution factor

% Protein = %Nitrogen x 6.25.

3.1.3.2 Crude Fibre Determination

An appropriate quantity of the sample (2.0g) was weighed into a conical flask, the sample was creamed by adding it slowly to 20ml of the cold 0.1275M H_2SO_4 before the remaining 180ml of the acid is added and brought to boiling point within 1minute

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(an appropriate amount of anti-foaming agent may be added if necessary). Boiling was done for 30 minutes by the use of a reflux condenser.

In the meantime, a Buckner funnel fitted with a perforated plate was prepared by adjusting a piece of cut cotton cloth or filter paper to cover the hole in the plate so as to serve as a support for a circular piece of suitable filter paper. At the end of the 30 minutes boiling period, the acid mixture was allowed to stand for 1minute and then poured immediately into a shallow layer of hot water under gentle suction in the prepared funnel. Washing of the insoluble matter was done with boiling water until the washing are free from acid, then wash back into the original flask by means of a wash bottle containing 200ml 0.175M NaOH solution measured at ordinary temperature and brought to boiling point. Boiling was done for 30minutes with the same precautions as those used in the earlier boiling and treatment. The whole of the insoluble material was transferred to the filter paper by means of boiling water, washed first with boiling water then with 1% HCl and finally with boiling H_2O until it is free of acid. Washing was done twice with alcohol and three times with ether.The residue was transfered to a dish (previously ignited and weighed) with boiling H_2O. After evaporating off the water on the water bath, the dish was dried in the 100^{0}C oven, weighed, Ignited in a muffle furnace at 500-550^{0}C to ash and reweighed.

Calculation

Weight of dish = ig

Weight of dish+sample before ashing = jg

Weight of dish+sample after ashing = kg

$$\% \text{ fibre} = \frac{j-k}{j-i} \times \frac{100}{1}$$

3.1.3.3 Crude Fat Determination

Oil was extracted from 10g of pulverized *L. purpureus* seeds in a Soxhlet extractor using hexane as a solvent. Solvent was removed in a rotary evaporator and the weight of the extracted fat was determined. Results were calculated as % fat in the original seed.

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$$\% \text{ Fat} = \frac{W_1 - W_0}{\text{Weight of sample}} \times \frac{100}{1}$$

W_0 = initial weight of dry Soxhlet flask

W_1 = final weight of oven dried flask + oil /fat

3.1.3.4 Ash Content Determination

A clean porcelain dish was ignited in the muffle furnace at 500-550°C for 1hour, cooled in a dessicator and weighed. An appropriate quantity (2.0g) of the sun-dried lablab bean seed was transferred into the porcelain dish and weighed. The porcelain dish with the sample was placed in the moisture extractor oven at 105°C for 2 hours and then transferred into a muffle furnace and set at the temperature of 250°C and gradually increased to 500°C or 550°C for 3-4 hours, to completely ash. After, the sample was removed from the muffle furnace, cooled in a dessicator and weighed.This was repeated until ashing was completed.

Calculation

Weight of porcelain dish = xg

Weight of porcelain dish + sample before ashing = yg

Weight of porcelain dish + sample after ashing = zg

$$\% \text{ Ash} = \frac{z - x}{y - x} \times \frac{100}{1}$$

3.1.3.5 Moisture Content / Dry Matter Determination:

Lablab bean seed (2.0g) sundried was weighed into a pre-dried, cooled and weighed percerlain dish (in triplicates) using Mettler electronic balance (Mettler PM 4000).

The dishes and its content were placed in a moisture extractor oven at 105°C for 4 hours. Using a pair of tongs, the dishes were transferred into a dessicator, allowed to cool and weighed. The dishes were returned to the oven for half an hour (30 minutes) and again it was cooled in the dessicator and weighed. This process was repeated until constant weight was achieved.

Calculation

Weight of dried porcelain dish = W_1

Weight of porcelain dish+ sample before drying = W_2

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Weight of porcelain dish+ sample after drying = W_3

$$\% \text{Moisture content} = \frac{W_2 - W_3}{W_2 - W_1} \times \frac{100}{1}$$

% Dry matter = 100 - %moisture

3.1.3.6 Gross Energy Determination

A small quantity (0.25g) of each sample was weighed into a steel capsule. A 10cm cotton thread was attached to the thermocouple to touch capsule. The bomb calorimeter was closed and charged in with oxygen up to 30 atmosphere. The bomb calorimeter was fixed up by depressing the ignite switch to burn the sample in an excess of oxygen. The maximum temperature rise in the bomb calorimeter was measured with the thermocouple and galvanometer system. The rise in temperature was compared with that obtained for 0.25g of benzoic acid standard of known caloric value.

The caloric value of each sample was determined by the following stepwise calculations.

Calculations

Mass of Benzoic Acid = W_1 g

Calorific value of 1g Benzoic Acid = 6.32kcal/g

Heat released from Benzoic Acid = 6.32 x Wkcal

Galvanometer deflection without sample = T_1

Galvanometer deflection of Benzoic acid = $T_2 - T_1$

$$\text{Calculation constant} = \frac{6.32 \times W_1}{T_2 - T_1} = y_1$$

It was standardized repeatedly five times and average value calculated for y.

Mass of sample = 0.25gm.

Galvanometer deflection with sample = T_3

Galvanometer deflection of sample = $T_3 - T_1$

Heat released from sample = $(T_3 - T_1)$ykcal

$$\therefore \text{Calorific value of sample} = \frac{(T_3 - T_1)\, y}{0.25} \text{ kcal/g}$$

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3.1.3.7 Nitrogen Free Extract (NFE): This is also known as the carbohydrate portion was calculated from the difference of all the other proximate parameters (excluding mineral content) from 100%.

% NFE = 100- (% M+ % CP+% CFt + % Ash + %CF).

3.1.4 Determination of Mineral Elements in Lablab seeds

The Sodium (Na) and potassium (K) contents were determined by flame photometry (Jenway Ltd, Dunmow Essex, UK) and phosphorus (P) by Vanado-molybdate method (AOAC, 1995). Calcium (Ca), Magnesium (Mg), Iron (Fe) and manganese (Mn) were determined after wet-digestion with a mixture of nitric, sulphuric and hydrochloric acids using atomic absorbtion spectrophotometry (Buck Scientific, East Norwalk, CT, USA).

3.1.5 Statistical analysis

The experiments were done in triplicates and the results expressed as standard error of mean. Data obtained were expressed as the mean ± standard error of mean (mean ± SEM). Significant differences between means were determined by the Student t-test (Bailey, 1992). Values of $p<0.05$ were considered statistically significant differences.

3.2 EXPERIMENT TWO

3.2.1 DETERMINATION OF THE ANTINUTRITIONAL FACTORS

3.2.1.1 DETERMINATION OF THE TRYPSIN INHIBITOR CONCENTRATION

PROCEDURE: Trypsin inhibitor activities were determined according to (Liener, 1979). 0.2g of sample was weighed into a screw-cap centrifuge tube. 10ml of 0.1M phosphate buffer were added and the contents were shaken at room temperature for 1 hour on a UDY shaker. The suspension obtained was centrifuged at 5000rpm for 5 minutes and filtered through Whatman No.42 filter paper. The volume of each was adjusted to 2 ml with phosphate buffer. The test tubes were placed in water bath, maintained at 37°C and 6ml of 5% tricarboxylic acid (TCA) solution were added to one of the tubes to serve as a blank. 2 ml of casein solution were added to all the tubes

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previously kept at 37°C and were incubated for 20 minutes. The reaction was stopped after 20 minutes by adding 6ml of TCA solution to the experimental tubes and then shaken. The reaction was allowed to proceed for 1 hour at room temperature. The mixture was filtered through Whatman No.42 filter paper and the absorbance of filtrate from sample and trypsin standard solutions were read at 280nm. The trypsin inhibitor concentration in mg/g sample was calculated, using the formula:

$$T.I.mg/g = \frac{\text{sample - blank}}{0.19 \times \text{sample wt in g}} \times \frac{\text{Dilution factor}}{1000 \times \text{sample size}}$$

3.2.1.2 DETERMINATION OF THE HEAMAGGLUTININ UNITS

PROCEDURE: Haemagglutinating unit was determined according to Jaffe, (1979). 2g of sample was weighed into 250 ml beaker. 50 ml of solvent of mixture of isobutyalcohol and trichloroacetic acid were added and allowed to shake on a UDY shaker for 6 hours to extract the haemagglutinin. The mixture was filtered through a double layer filter paper into a 250 ml conical flask and maintained in a water bath for 2 hours at 80°C. The filtrate was allowed to cool. A set of standard solutions of haemagglutinin ranging from 0ppm to 10ppm were prepared from the haemagglutinin stock solution. The absorbance of the standard solution as well as that of the filtrate was read at 220 nm on a digital spectrophotometer 21D.

Heamagglutinin was calculated using the formula

Heamagglutinin HU/mg =Absorbance of sample x Average of gradient standard x Dilution factor

3.2.1.3 DETERMINATION OF CYANOGENIC GLYCOSIDE (HYDROCYANIDE) CONTENT

PROCEDURE: Cyanogenic glycosides (HCN) was determined according to Bradbury *et al.*, (1999). 5 g of each sample was weighed into 250 ml conical flask and each sample was incubated for another 16 hours at a temperature of 38°C. After the extraction, the filtration was done using a double layer of hardened filter paper. The distillation was done with Markham distillation apparatus. Each sample extracted was transferred into a two-necked 500 ml flask connected with a steam generator. This was steam-distilled with saturated sodium bicarbonate solution contained in a 50 ml conical

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flask for 60 minutes. 1 ml of starch indicator was added to 20 ml of each distillate and was titrated with 0.2N of iodine solution. The percentage hydrocyanide was calculated with the formula:

$$\text{Hydrocyanide (mg/g)} = \frac{\text{Titre} \times 10 \times 0.27 \times 1000}{\text{weight of sample}}$$

3.2.1.4 DETERMINATION OF THE OXALIC ACID (OXALATE) LEVEL

PROCEDURE: Oxalate level was determined according to Fassel (1996). The extraction was done by weighing 1g of each sample into 250 ml conical flask and soaked with 100 ml of distilled water. These were allowed to stand for 3 hours and each was filtered through a double layer of filter paper. 10 ppm, 20 ppm, 30 ppm, 40 ppm and 50 ppm standard solution of oxalic acid were prepared and read on the spectrophotometer at 420 nm for the absorbance. The absorbance of filtrate from each sample was also read on the spectronic 20. Percentage oxalate was calculated using the formula:

Oxalate (mg/g) = Sample absorbance x Average gradient from the curve for standard x Dilution factor

3.2.1.5 DETERMINATION OF THE PHYTIC ACID (PHYTATE) LEVEL

PROCEDURE: Phytate level was determined according to Maga, (1983). 2g of each sample was weighed into 250 ml conical flask. 100ml of 2% concentrated hydrochloric acid was used to soak each sample into conical flask for 3 hours. This was filtered through a double layer of hardened filter paper. 50ml of each filtrate were placed in 250 ml beaker and 107 ml of distilled water were added in each case to give proper acidity. 10ml of 0.3% ammonium thiocyanate solution were added into each solution as indicator. This was titrated with standard iron (III) chloride solution which contained 0.00495g iron per ml. The end point was slightly brownish-yellow which persisted for 5 minutes. The percentage phytic acid was calculated using the formula:

$$\text{Phytic acid (mg/g)} = \frac{X \times 1.19 \times 3.55}{2}$$

where X = Titre value x 0.00195

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3.2.1.6 DETERMINATION OF THE TANNIC ACID (TANNIN) LEVEL

PROCEDURE: Tannin level was determined according to Dawra *et al.* (1988). 0.2g of each sample was weighed into a beaker. Each was soaked with solvent mixture; 80 ml of acetone and 20 ml of glacial acetic acid for 5 hours to extract tannin. The filtrates were removed. The samples were filtered through a double layer filter paper to obtain the filtrate. A set of standard solution of tannic acid was prepared ranging from 0 ppm to 10 ppm. The absorbances of the standard solution as well as that of the filtrates was read at 720 nm on a spectronic 20. The percentage tannin was calculated using the formula:

Tannin mg/g = Absorbance x Average gradient x Dilution factor

3.2.1.7 DETERMINATION OF THE SAPONIN ($C_{30}H_{20}O_{10}$) CONTENT

PROCEDURE: Saponin level was determined according to Brunner (1984). 2g of sample was weighed into a 250 ml beaker and 100 ml of isobutylalcohol (octanol) was added and left for 5 hours on a UDY shaker for uniform mixing to obtain a uniform solution. The mixture was then filtered through a No. 1 Whatman filter paper. The filtrate is transferred to another 100ml beaker and was saturated with magnesium carbonate solution. The mixture was transferred into 100 ml volume flask and made up to mark with distilled water. The mixture obtained here was then filtered to obtain a clear colourless solution to be read on a spectrophotometer at 380 nm.

0 ppm to 5 ppm of standard saponin solutions were prepared from 1000 ppm saponin stock standard solution and saturated with magnesium carbonate as above and also filtered. The absorbances of the saponin standard solution (i.e. 0-5 ppm) were also read at 380 ppm to obtain the gradient of plotted curve.

$$\text{Saponin mg/g} = \frac{\text{Absorbance Std.-Absorbance Sample} \times \text{Dil Factor} \times \text{Av. gradient}}{\text{Weight of sample}}$$

3.2.1.8 DETERMINATION OF THE TOTAL ALKALOIDS LEVEL

PROCEDURE: Alkaloid level was determined according to Henry (1973).

2 g of sample was weighed into a 100 ml conical flask and 20 ml of 80% alcohol added to give a smooth paste. The mixture was transferred to a 250 ml flask and more alcohol was added to give up to 100 ml. 1g of magnesium oxide was added. The mixture was digested in a boiling water bath for 1.5 hours under a reflux air condenser with

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occasional shaking. The mixture was filtered while hot through a small Buchner funnel. The residue was returned to the flask and redigested for 30 minutes with 50 ml alcohol after which the alcohol will be evaporated, adding hot water to replace the alcohol lost. When all the alcohol has been removed, 2 to 3 drops of 10% HCl were added. The whole solution was later transferred into a 150 ml volumetric flask. 5 ml of zinc acetate solution and 5 ml of potassium ferrocyanide solution were added, thoroughly mixed to give a homogenous solution.

The flask was allowed to stand for a few minutes, filtered through a dry filter paper and 10 ml of the filtrate were transferred into a separating funnel and the alkaloids present were extracted vigorously by shaking with five successive 30 ml portions of chloroform. The residue obtained was dissolved in hot water and transferred into a Kjeldahl flask with the addition of 0.2 g sucrose and 10ml conc. H_2SO_4 and 0.02g selenium for digestion to a colourless solution. The % NH_3 was determined by Kjeldahl distillation method; the %nitrogen is converted to a %total alkaloid by multiplying by a factor of 3.26.

3.2.2 PHYTOCHEMICAL ANALYSIS: METHODOLOGY

Phytochemical analyses of both the aqueous and the methanolic extracts of Lablab beans were conducted, using the method of Trease and Evans (1983), as follows:

1. Test for Flavonoids: 0.5 g of each extract in separate test-tubes were added some magnesium ribbons and 5 ml of concentrated HCl and were observed for red colourations.

2. Test for **Saponins**: 0.5 g of each extract was shaken vigorously with hot distilled water in separate test-tubes and were observed for persistence frothings.

3. Test for **Tannins**: 0.5 g of each extract was boiled with 10 ml of distilled water for 15 minutes, filtered and made up to 10ml with distilled water. To 2 ml of each filterate was added 10ml of distilled water and one drop of $FeCl_3$ solution and observed for blue/green colourations.

4. Test for **Phlobatannins**: 5 ml of the filterates from 3 above, 3 drops of 4 % formaldehyde and 6 drops of dilute HCl solution were added. The temperature of each mixture was raised to boiling point, and then cooled. The bulky precipitates formed were

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washed with hot distilled water, ethanol and warm 5%(w/v) KOH solution, successively. The residues were observed for appropriate colourations.

5. **Test for Cardiac Glycosides:** 0.5g of each extract was mixed with $CHCl_3$ in test-tubes and H_2SO_4 was carefully added to form a layer. The interfaces were observed for reddish-brown colourations.

6. **Test for Alkaloids:** 25mg of each extract was dissolved in distilled water and filtered. The filterates were acidified with 1M HCl and 1ml of each filterate was treated with 2 drops of Mayer's reagent and were observed for formation of precipitates. Another 1ml from each filterate was treated with Dragendorff's reagent and was observed for the formation of precipitates or turbidity.

3.3 EXPERIMENT THREE

3.3.1 Nutritional Evaluation of Lablab beans

Five diets were prepared for the study. These included a protein-free (basal) diet, a standard (reference) diet, which served as control diet and the three test diets containing the three varieties of the *L. purpureus* seeds. Parameters employed in determining the nutritive quality of the lablob seeds included Protein efficiency ratio (PER), net protein ratio (NPR), protein retention efficiency (PRE) and feed conversion efficieny (FCE). The standard reference protein diet was prepared according to Sarwatt *et al.*, (1991).

The composition of the standard reference diet and basal protein-free diet are given in Table 4

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Table 4 Composition of Standard Reference and Basal Protein-free diet

Composition of standard reference diet		Basal Protein-free diet
Ingredient	%	%
Maize meal	48	0
Maize bran	12.9	0
Corn starch	0	70
Groundnut cake	9.2	0
Sucrose	0	15
Groundnut oil	0	5
Soybean meal	25	0
Fish meal	2.5	0
Mineral	2.0	4
Vitamin / trace Mineral premix	0.1	1
Salt	0.3	0
Cellulose	0	5

Vitamin trace mineral premix supplied per kg of diet.

Vit.A (10,000 I.U), vit D_3 (2000I.U), vit E (5I.U), vit K(2.24mg), riboflavin (55mg), pantothenic acid (10mg), nicotinic acid(25mg), choline (350mg), folic acid (1mg), methionine (450mg), Mn (56mg), I (1mg), Fe (20mg), Cu (10mg), Zn (50mg), Co (1.25mg). Sarwatt *et al.* 1991.

Maize meal and maize bran were the sources of energy while soyabean meal and fishmeal served as protein sources.

3.3.2 Assessment of the Nutritional Quality of the Diets

The following criteria for protein utilization were worked out on the basis of analysis (for nitrogen content) of the diets and faeces respectively.

1. Protein Efficiency Ratio (PER): It was done according to Pellet and Young, (1980). This method expresses the protein efficiency ratio as the gain in body weight per gram of protein eaten over the experimental period.

$$PER = \frac{\text{Weight gain of test animal (g)}}{\text{Protein consumed (g)}}$$

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2. Net Protein Ratio (NPR): This was done by the method of Bender and Doell, (1957).

$$NPR = \frac{\text{Weight gain of test protein group} + \text{weight loss of the N-free diet group}}{\text{Protein intake (g)}}$$

3. Protein Retention Efficiency (PRE): This was computed as NPR value x 16 Bender and Doell, (1957).

4. Feed Conversion Efficiency/Ratio (FCE): This was computed as:

$$\frac{\text{weight gain/loss}}{\text{feed intake (g)}}$$ Agunbiade (1992)

3.4 EXPERIMENT FOUR

3.4.1 Toxicological studies associated with ingestion of *Lablab purpureus* seeds in rats

Materials and Methods

Twenty weanling male albino rats of the Wistar strain obtained from the rat colony of the Department of Physiology, College of Medicine, University of Ibadan, were used for this study. The rats with average body-weights of about 165.2±8.2g were randomly selected and housed in stainless-steel individual metabolic cages (Associated Crate Ltd. England) located at the Animal House of the Department of Animal Science, University of Ibadan. Five rats were put on each test diet. The experimental diets contained raw lablab beans; Rongai white, Rongai brown, and Highworth black varieties obtained from I.I.T.A., Ibadan. The rats in the control group were fed rat pellets manufactured by Bendel Feeds and Flour Mills Ltd. Edo State. The composition of the control diet is given below in Table 5.

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Table 5: Composition of feed for rats in control group

Ingredient	%
Protein	21
Fat	3.4
Fibre	6.0
Calcium	0.8
Phosphorus	0.8
Carbohydrate	67
Vitamins	1.0

3.4.1.1 Blood Collection and Preparation

At the end of the experiment, about 6ml of blood was collected from the rats through the orbital veins. 2ml of the blood from each rat were put into bottles containing sodium ethylene diamine tetraacetic acid (Na_2EDTA) and used to determine haematological parameters. Another 4ml of blood from each rat were collected into a sample bottle for serum biochemical analysis.

The blood for serum analysis was allowed to clot at room temperature in the clean sample bottles and then centrifuged for 10 minutes in a bench centrifuge at 2000 r.p.m. The clear serum was siphoned into clean sample bottles and stored immediately in the freezer until required for analysis. The sera separated from the clot by centrifugation were used to determine serum biochemical parameters.

2ml blood for whole blood count (red blood cell count and white blood cell count) were collected into clean EDTA bottles as anticoagulant to avoid clotting and the samples were immediately analysed by the microhaematocrit method of Jain (1986).

Packed Cell Volume (PCV)	Microhaematocrit
Haemoglobin concentration (Hb)	Cynamethaemoglobin
Red Blood Cell count (RBC)	Haemocytometer
White Blood Cell count (WBC)	Haemocytometer
Differential Leucocyte Count (DLC)	Giemsa staining

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3.4.2. ERYTHROCYTE VALUES

3.4.2.1 Packed Cell Volume (PCV)

The PCV was determined using the microhaematocrit method as described by Jain (1986). In this method blood was drawn into the plain capillary tube by capillary traction to ¾ of its length. The capillary tube was tipped to permit the blood to flow toward the free end to provide sufficient space to prevent outflow when the opposite end was sealed. The outside of the capillary tube was wiped free of blood as the index finger was placed over the moist end to hold the column of blood in place as the opposite dry end was forced into the sealing material (plasticine) to form a tight plug. The capillary tube was placed in a microhaematocrit centrifuge (Hawksley, Gelman instruments, England) with the sealed end pointing outward. The blood was then centrifuged for 5 minutes at a speed of 3,000 r.p.m. PCV was then determined on a graphic reading scale.

3.4.2.2 Haemoglobin (Hb) Concentration

The blood haemoglobin concentration was estimated by the cyanmethaemoglobin method as described by Schalm *et al.* (1975). Drabkin's solution was prepared by placing 500 ml of water in a 1 litre volumetric flask and 1g of sodium bicarbonate ($NaHCO_3$), 0.05g potassium cyanide (KCN) and 0.02g potassium ferricyanide ($K_3Fe(CN)_6$) were added. The content was dissolved and distilled water added to make the total volume to 1 litre.

The procedure for haemoglobin determination is as follows: 4ml of Drabkin's solution were carefully transferred to a clean test tube. Then 0.02ml of blood was transferred into the test tube using the multichannel micropipette. The pipette was rinsed out several times. This gave a blood to reagent dilution of 1:200. The mixture was then left for 10 minutes for cyanmethaemoglobin to form. The optical density (O.D) was then read using a spectrophotometer (SP 6100 model, Jenway, England), at a wavelength of 540nm, using 4ml of Drabkin's solution as the blank. The optical density (OD) of a haemoglobin standard of known concentration was then determined and the value used to calculate haemoglobin concentration in the sample thus:

$$\text{Haemoglobin (Hb) conc. (g/dl of blood)} = \frac{\text{O.D. of blood}}{\text{O.D. of standard}} \times \text{Hb. conc. of std.}$$

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3.4.2.3 Erythrocyte (RBC) COUNT

The RBC count was made in a haemocytometer using Hayem's solution (0.5 mercuric chloride, 1.0g sodium chloride, 5g sodium sulphate and 200ml distilled water) as the diluent. The number of erythrocytes in 5 of the 25 squares in the central area of each chamber of the Neubauer haemocytometer were counted, taking the 4 corners squares and the central one.

The total number of erythrocytes obtained was multiplied by depth (x10), area (x5) and dilution factor (x200). Hence if Y erythrocyte had been counted, the number of erythrocytes per millimeter of blood in the original sample would be 10,000 Y.

3.4.2.4 Haematometric Indices

Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were calculated from the values of RBC, PCV and Hb as described below.

MCV provides the average volume of the individual RBC

$$MCV\ (fl) = \frac{PCV / 100 \times 1000}{RBC\ (x10^6/\mu L)}$$

MCH expresses the average weight of Hb present in an erythrocyte

$$MCH\ (pg) = \frac{Hb\ (g/dl) \times 10}{RBC}$$

MCHC gives the percentage of the MCV which the Hb occupies.

$$MCHC\ (g/dl) = \frac{Hb\ (g/dl) \times 100}{PCV}$$

3.4.2.5 Total and Differential Leucocyte (White Blood Cells) Values

Total WBC counts were made in duplicates in a haemocytometer using the white blood cell diluting fluid. The sum of WBC counts from 4 large corner squares of the haemocytometer chamber was multiplied by the depth (x10) and the dilution factor (x20) and divided by the number of squares counted. Thus the number of leucocytes per millimeter in the original samc was 50x, where x was the number of leucocytes counted in the 4 squares.

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Differential WBC counts in which 100 cells per slide were counted, were made from Giemsa stained blood smears.

3.4.2.6 HISTOLOGICAL EXAMINATION

The histological examination of the tissues was carried out by processing and staining the tissues according to the method of Raghuramulu *et al.* (1993).

The slides were prepared as follows:

a. **Fixation:** Pieces of tissue from the organs were fixed in 10% formalin solution to preserve tissue morphology and chemical composition.

b. **Dehydration:** Tissues were dehydrated after fixation in graded series of ethanol ranging from 70 to 100%.

c. **Clearing:** Tissues were cleared in xylene to impregnate them with paraffin solvent.

d. **Infilteration:** Tissues were infilterated for 2 hours in 50% v/v of xylene and ethanol.

e. **Embedding:** The tissues were embedded in molten paraffin wax and placed in the oven at a temperature of 56-58^{0}C to allow penetration of paraffin wax into the intercellular spaces of tissues so as to facilitate sectioning.

f. **Cutting and sectioning:** Small blocks of paraffin containing the tissues were then sectioned using the blade of a microtone adjusted for cutting 5µ sections.

g. **Mounting and staining:** These sections were then placed in warm water before they were transferred to clean slides and stained with haematoxylin and counter stained with eosin in this order- immersion in absolute ethanol, 70% ethanol, water and haematoxylin, water and 70% ethanol again, then eosin, 80% ethanol and lastly absolute ethanol. The prepared slides were viewed under the microscope.

Photomicrographs of the lesions were taken using ortholux microscope fitted with a Leitz camera unit and processed routinely in a colour photo laboratory.

3.4.2.7. Determination of Gamma-glutamyltransferase activity (GGT):

The principle is that Gamma-glutamyltransferase transfers the γ-glutamyl group of L-γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine. The amount of 5-amino-2-

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nitrobenzoate liberated is proportional to the GGT activity and can be measured photometrically.

L-γ-glutamyl-3-carboxy-4-nitroanilide + glycylglycine — γGT— L-γ-glutamyl-glygylglycine + 5-amino-2-nitrobenzoate

Reagents-working solutions

1. TRIS buffer: 123mmol/L, pH 8.25 (25 ^{o}C)
   Glycylglycine: 123mmol/L; preservative;additive
2. Acetate buffer: 10mmol/L, pH 4.5 (25 ^{o}C)
   L-Y-glutamyl-3-carboxy-4-nitroanilide: 25mmol/L, stabilizer, preservative.

TRIS = tris(hydroxymethyl)-aminomethane

3.4.2.7.1. Determination of Serum total protein.

This is based on the Biuret reaction as described by Gornall *et al.* (1949).

Principle: The peptide linkages of the amino acids making up a protein molecule are capable of reacting with copper in alkaline solution to produce a violet colour. This is the popular Biuret reaction of peptides and proteins.

Reagents

Preparation of the Biuret reagent: 3g of $CuSO_4.5H_2O$ and 6g of sodium-potassium tartrate were all dissolved in 500ml of distilled water. 300ml of 20% NaOH solution were then added with constant stirring and the final solution was made up to 1 litre with distilled water in a standard volumetric flask.

Standard Bovine Serum Albumin (BSA) Solution (1mg/ml): 50mg of bovine serum albumin crystals were dissolved in 50ml of warm distilled water in a standard volumetric flask.

Method: Serum sample (0.1ml) In a clean test tube was added 2.9ml of distilled water (test). In another clean test-tube was placed 3ml of bovine serum albumin (BSA) which was the standard protein; and 3ml of distilled water were placed in another test-tube as the blank. To each of these test-tubes, was added 3ml of the Biuret reagent and were warmed at 37°C for 10 minutes. The tubes were allowed to cool and the optical densities of the resulting violet solutions were read at 540nm in a spectrophotometer. The protein content was calculated in each case using the equation:

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Concentration of Test = $\frac{\text{Optical density of Test}}{\text{Optical density of Standard}}$ x Conc. of Standard
(mg/100ml)

3.4.2.7.2 Determination of Serum Albumin Concentrations

This was determined using the modification of Bromocresol green method of McPherson and Everad (1972).

Principle: The dye Bromocresol green at a pH below the isoelectric point of albumin reacts with albumin to cause a change in colour which is proportionable to the amount of albumin present.

Reagents

1. **Molar Glycine Solution:** This was prepared by dissolving 75.07g glycine in 100ml distilled water in a standard volumetric flask that was stored in the refrigerator until required.
2. 1m HCl: 8.8ml of concentrated HCl were distilled to 1 litre with distilled water.
3. Bromocresol green (0.02M): This was prepared by dissolving 1.39g of crystals of bromocresol green dye in 100ml of absolute alcohol. The solution was then stored in a dark bottle at room temperature.
4. **The Working Colour Reagent:** This was prepared by adding 94.5ml of the molar glycine solution to 800ml of distilled water. 5.5ml of 1M HCl were then added, followed by 3ml of the 0.02M bromocresol green solution. The resulting solution was then made up to 1 litre with water in a volumetric flask and stored in a refrigerator.
5. **Standard Albumin Solution:** This was prepared by dissolving 100mg of egg-albumin in 100ml of distilled water, giving a concentration of 1mg/ml.

Method: 5ml of the alcoholic solution of the bromocresol green (dye/colour) reagent was added to 0.02ml of serum; the colour change was read immediately against a reagent blank in a spectrophotometer at 635nm using an albumin standard solution; albumin concentrations were calculated with the equation:

Total Albumin (mg/100ml) = $\frac{\text{Optical density of Test}}{\text{Optical density of Standard}}$ x Conc. of Standard

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3.4.2.7.3 Determination of Serum Globulin

The globulin level in the serum was calculated by simple approximation, since the total serum protein level is the addition of the albumin and globulin fractions.

Therefore, Globulin concentration= Total protein - Albumin concentration

The determination of the other serum biochemical assays (AST, ALT, ALP, Urea and Creatinine are reported below in Experiment Five.

3.5 EXPERIMENT FIVE

Free Radical Scavenging / Anti-oxidant Activities of the Anti-nutritional Factors in Three varieties of Lablab purpureus seeds

MATERIALS AND METHODS

3.5.1 ANIMALS AND MANAGEMENT

Twenty male Wistar rats obtained from the Animal House of the Faculty of Veterinary Medicine, University of Ibadan, Ibadan weighing 206.25 ± 22g, were housed in a well-ventilated animal house of the Department of Biochemistry, University of Ibadan, Ibadan. The animals were provided with rat pellets, unlimited water and subjected to natural photoperiod of about 12 hours light and 12 hours darkness daily. The animals were acclimatized to the environment for 7 days before the commencement of treatment. Animals were then randomly numbered and divided into four equal groups of eight rats per group. 14 days after feeding, the rats were killed by cervical dislocation. The liver, kidney, testes and epididymes, were excised and organs' weights recorded. Fresh caudal epididymis was processed to determine the sperm counts. Aliquots of sperm suspension were stained in eosin and smears were examined for abnormal sperms. Samples from the testes were fixed in Bouin's fixative and processed for histopathological examination.

The percentage feed composition of the test diets and control diet are shown in Table 6 below. The feed was formulated to provide isoproteinic (20%) and isocaloric (3Kcals) ration for each rat.

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Table 6 % Feed Composition of rats for Antioxidant Studies

Ingredients	Control %	Rongai Brown %	Rongai White %	Highworth Black %
Casein	20	-	-	-
Lablab bean meal	-	82.8	86.6	87.7
Corn starch	15	3.6	1.7	1.15
Sucrose	50	-	-	-
Corn oil	5.0	3.6	1.7	1.15
Cellulose	5.0	5.0	5.0	5.0
DL Methionine	0.3	0.3	0.3	0.3
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1.0	1.0	1.0	1.0
Choline chloride	0.2	0.2	0.2	0.2
Total %	100	100	100	100

Vitamin A 10,000,000 I/U/2.5kg; Vitamin D_3 2,000,000 IU/2.5kg; Vitamin E 12,000 mg/2.5kg; Vitamin K_3 2000 mg/2.5kg; Vitamin B_1 1,500 mg/2.5kg; Vitamin B_2 5000 mg/2.5kg; Vitamin B_6 1,500 mg/2.5kg; Vitamin B_{12} 10mg/2.5kg; Niacin 15,000mg/2.5kg; Calpan 5000mg/2.5kg; Folic acid 600mg/2.5kg; Biotin 20mg/2.5kg; Choline Chloride 150,000mg/2.5kg; Manganese 80,000mg/2.5kg; Iron 40,000mg/2.5kg; Zinc 60,000mg/2.5kg; Copper 8,000mg/2.5kg; Iodine 1000mg/2.5kg; Cobalt 250mg/2.5kg

3.5.2 Preparations of tissues for biochemical analysis and histopathological examination

All the tissues collected after sacrifice were washed in ice-cold 1.15% KCl, blotted and weighed. The liver, kidney and testes were then homogenized in 4 volumes of the homogenizing buffer (pH, 7.4) using a Teflon homogenizer. The resulting homogenate was centrifuged at 10,000g, 4°C for 10 minutes. The supernatant was collected and processed for biochemical estimations. Samples from the testes were cut into two pieces, fixed in Bouin's fixative, sectioned and stained routinely with hematoxylin and eosin for histopathological examination.

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Reagents

1. **Washing buffer (1.15% Potassium chloride)**

   1.15g of potassium chloride (BDH Chemical Limited, England) was dissolved in distilled water and made up to 1000ml and stored at 4°C.

2. **Homogenizing buffer (50mM Tris-HCl, 1.15% KCl, pH 7.4)**

   7.80g of Tris(hydroxymethyl)aminoethane (Sigma chemical Co., St Louis U.S.A) and 11.5g of potassium chloride were dissolved in 900ml of distilled water. The pH was adjusted to 7.4 and then made up to 1 litre with distilled water.

3.5.2.1 PROTEIN DETERMINATION

Protein concentrations of the liver, kidney and testes homogenate were determined by Biuret reaction as described by Gornal *et al.*, (1949), with modification: potassium iodide was added to the Biuret reagent to prevent the precipitation of Cu^{2+} as cuprous oxide.

Principle

Cu^{2+} ions in alkaline pH form complexes with protein as exemplified by the Biuret reagent containing copper II sulphate ($CuSO_4$), potassium iodide (KI) and sodium potassium tartrate ($KNaC_4H_4O_6.4H_2O$). The protein and Biuret reagent form blue complexes with maximum absorbance at 540nm. The procedure is usually calibrated with a standard BSA curve.

Reagents

(a) 0.2M Sodium Hydroxide

8g of NaOH (BDH, England) was dissolved in distilled water and the solution made up to 1 litre.

(b) Biuret Reagent

3g of copper sulphate $CuSO_4.5H_2O$ (BDH Chemicals Limited England) and 9g of Na-K tartrate were dissolved in 500ml of 0.2M NaOH. 5g of potassium iodide (KI) was added and the solution made up to a litre with 0.2M NaOH.

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(c) **Stock Bovine Serum Albumin (standard)**

20 mg of BSA (Sigma Chemical Co., USA) was dissolved in 2 ml distilled water to give a stock solution of 10 mg protein/ml.

Standard BSA curve by the Biuret Method

Several dilutions of the stock solution containing 2mg to 10mg protein/ml were made. Into 1ml of each protein standard solution in a test tube was added 4ml of Biuret reagent. The mixture was allowed to stand at room temperature for 30 minutes and the optical densities of the resulting oily solutions were read in a spectrophotometer at 540nm. A curve of the optical density against protein concentration was plotted.

Determination of Protein in the Samples

Procedure

The post mitochondrial fractions of the liver, kidney and testicular supernatants were diluted 100 times with distilled water. This was done to reduce the sensitivity range of Biuret method. 1ml of the diluted sample was added to 3ml of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540nm using distilled water as blank. The protein content of the samples were usually extrapolated from the standard curve and multiplied by 100 to get the actual amount in the fraction.

3.5.2.2 ESTIMATION OF REDUCED GLUTATHIONE (GSH) LEVEL

The method of Beutler *et al.*, (1963) was followed in estimating the level of reduced glutathione (GSH).

Principle

The reduced form of glutathione comprises in most instances the bulk of cellular non-protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable yellow colour when [5',5' -dithiobis- (2-nitrobenzoic acid) (Ellman's reagent) DTNB] is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman reagent with the reduced glutathione, 2-nitro-5-thiobenzoic acid possesses a molar absorption at 412 nm. Reduced GSH is proportional to the absorbance at 412 nm.

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This method has the following advantages over the earlier modification of the nitroprusside method.

i. The precipitation process is carried out with a single easily prepared reagent. It does not require addition of solid sodium chloride or prolonged shaking.

ii. The determination may be carried out at any temperature likely to be encountered in the laboratory.

iii. The colour formed is relatively stable.

iv. The reagent for colour development is stable for many weeks.

v. The sensitivity of the method is so good that it may readily be adapted to a micro-procedure.

GSH + Ellman's reagent → Nitro 5-thiobenzoate + GSS-

Figure 3: Reaction of reduced GSH with Ellman's reagent

Reagents

1. GSH working standard

40mg GSH (Sigma, Mol. Weight 307.3g) was dissolved in 100ml of 0.1M phosphate buffer, pH 7.4, and then stored in the refrigerator.

2. 0.1 M Phosphate Buffer (pH 7.4).

a. 0.1M $Na_2HPO_4.12H_2O$ (MW=358.22) was prepared by dissolving 7.16g in 200ml of distilled water.

b. 0.1M $NaH_2PO_4.2H_2O$ (MW=156.03) was prepared by dissolving 1.56g in 100ml of distilled water.

Finally, 0.1M phosphate buffer was prepared by adding (a) to (b) and the pH adjusted to 7.4 with drops of concentrated HCl or NaOH as the case may be.

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3. **Ellman Reagent [5',5'- Dithiobis- (2-nitrobenzoate) DTNB].**

This was prepared by dissolving 120mg (1.2g) of Ellman reagent in 0.1M phosphate buffer and made up to 100ml.

4. **Precipitating Agent**

4% sulphur-salicylic acid ($C_7H_6S.2H_2O$ Mol. Wt. 254.22) was prepared by dissolving 2g of sulphur-salicylic acid in 50ml of distilled water. This is stable for approximately three weeks at 4°C.

CALIBRATION OF GSH STANDARD CURVE

PROCEDURE

Preparation of the GSH Standard Curve

GSH is proportional to absorbance at 412nm. All readings were taken within 5 minutes, as the colour developed is not stable after that duration, following addition of Ellman reagent.

Protocol

An aliquot of the liver, kidney and testes homogenates were deproteinised by the addition of an equal volume of 4% sulfosalicylic acid. This was centrifuged at 4000g for 5 minutes. Thereafter, 0.5 ml of the supernatant was added to 4.5 ml of Ellman reagent. Reduced glutathione, GSH, is proportional to the absorbance at 412 nm.

3.5.2.3 ESTIMATION OF GLUTATHIONE PEROXIDASE ACTIVITY

This was done according to the method of (Rotruck *et al.*, 1973).

Reagents

1. **0.1 M Phosphate Buffer (pH 7.4).**

a. 0.1M $Na_2HPO_4.12H_2O$ (MW=358.22) was prepared by dissolving 7.16g in 200 ml of distilled water.

b. 0.1M $NaH_2PO_4.2H_2O$ (MW=156.03) was prepared by dissolving 1.56g in 100 ml of distilled water.

Finally, 0.1M phosphate buffer was prepared by adding (a) to (b) and the pH adjusted to 7.4 with drops of concentrated HCl or NaOH as the case may be.

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2. **Sodium Nitrite (NaN_3 10mM)**

0.03g of sodium nitrite was dissolved in 50 mls of distilled water.

3. **Reduced Glutathione (GSH 4mM)**

0.02g of reduced GSH was dissolved in 20 mls of phosphate buffer.

4. **Hydrogen peroxide (H_2O_2 2.5mM)**

28µL of hydrogen peroxide was dissolved in 100 mls of distilled water.

5. **Trichloroacetic acid (TCA) (10%)**

4g of TCA was dissolved in 40 mls of distilled water.

6. **Di-potassium hydrogen orthophosphate (K_2HPO_4) 0.3M**

5.23g of di-potassium hydrogen orthophosphate was dissolved in 100 ml of distilled water.

7. **5'-5'-dithlobis(2-diniltrobenzoic acid) [DTNB]**

0.04g of DTNB was dissolved in 100 ml of phosphate buffer.

The whole reaction mixture was incubated at 37°C for 3 minutes after which 0.5ml of TCA was added and thereafter centrifuged at 3000rpm for 5 minutes. To 1 ml of each of the supernatants, 2ml of K_2HPO_4 and 1ml of DTNB were added and the absorbance was read at 412 nm against a blank.

CALCULATION

Glutathione peroxidase activity was observed by plotting the standard curve and the concentration of the remaining GSH was extrapolated from the curve.

GSH consumed = 245.34 - GSH remaining

$$\text{Glutathione peroxidase activity} = \frac{\text{GSH consumed}}{\text{mg protein}}$$

3.5.2.4 ESTIMATION OF GLUTATHIONE-S-TRANSFERASE ACTIVITY

The activity of glutathione-S-transferase (GST) was determined by the method of Habig *et al.* (1974).

Principle

It is based on the fact that all known glutathione-S-transferase demonstrate a relatively high activity with 1-chloro-2,4-dinitrobenzene as the second substrate. Consequently, the conventional assay for Glutathione-S-transferase activity utilizes 1-

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chloro-2,4-dinitrobenzene as substrate. When 1-chloro-2,4-dinitrobenzene is conjugated with reduced glutathione, its maximum absorption shifts to a longer wavelength of 340 nm provides a direct measurement of the enzymatic reaction.

Reagents

1. 0.1 M Phosphate Buffer (pH 6.5).

a. 0.1M $Na_2HPO_4.12H_2O$ (MW=358.22) was prepared by dissolving 7.16g in 200 ml of distilled water.

b. 0.1M $NaH_2PO_4.2H_2O$ (MW=156.03) was prepared by dissolving 1.56g in 100 ml of distilled water.

Finally, 0.1M phosphate buffer was prepared by adding (a) to (b) and the pH adjusted to 6.5 with drops of concentrated HCl.

2. 20mM 1-Chloro-2,4-dinitrobenzene (CDNB)

0.03g of 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma Chemical Co., London) was dissolved in 10ml ethanol.

3. 0.1M Reduced Glutathione

61.46mg glutathione, GSH (Sigma Chemical Co., London) was dissolved in 2ml of 0.1M phosphate buffer of pH 6.5.

Assay Protocol

The reaction was allowed to run for 3 minutes (180 seconds) and absorbance was read at 60 seconds-interval against the blank at 340 nm wavelength. The temperature was maintained at approximately 31°C.

The activity of glutathione-S-transferase activity was computed with the molar extinction coefficient of CDNB at 340nm wavelength = $9.6mm^{-1}cm^{-1}$.

$$\text{GST activity} = \frac{\text{Absorbance/min}}{\text{CDNB, 340nm}} \times \frac{1}{0.03\text{ml/mg protein}} = \mu\text{mole/min/mgprotein}$$

3.5.2.5 ASSESSMENT OF LIPID PEROXIDATION

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation.

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Principle

This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde: an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 532nm and which is extractable into organic solvents such as butanol. Malondialdehyde (MDA) is often used to calibrate this test and thus the results are expressed as the amount of free MDA produced.

2 Thiobarbituric acid (TBA) + Malondialdehyde (MDA) → MDA-TBA$_2$ (Pink coloured complex)

Figure 4: MDA reaction in lipid peroxidation assay

Reagents

1. **0.15M Tris-KCl Buffer (pH 7.4).**

   1.344g KCl and 2.832g Tris base were dissolved separately in distilled water and made up to 100ml with same. The pH was then adjusted to 7.4.

2. **30% Trichloroacetic acid**

   12g TCA (CCl_3COOH) was dissolved in distilled water and made up to 40ml with same.

3. **0.75% Thiobarbituric acid (TBA)**

   This was prepared by dissolving 0.3g of TBA in 0.1M HCl and made up to 40ml with same.

PROCEDURE

An aliquot of 0.4ml of the samples was mixed with 1.6ml of Tris-KCl buffer to which 0.5ml of 30% TCA was added. Then 0.5ml of 0.75% TBA was added and placed in a water bath for 45 minutes at 80°C. This was then cooled in ice and centrifuged at 3000g for 15 minutes. The clear supernatant was collected and absorbance measured

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against a reference blank of distilled water at 532nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi, (1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of

$1.56 \times 10^5 M^{-1} cm^{-1}$.

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532nm} \text{ x volume of sample x mg protein}}$$

3.5.2.6 HYDROGEN PEROXIDE GENERATION

Hydrogen peroxide generation was determined according to the method of Wolff (1994).

Reagents

1. **0.1 M Phosphate Buffer (pH 7.4).**

   a. 0.1M $Na_2HPO_4.12H_2O$ (MW=358.22) was prepared by dissolving 7.16g in 200ml of distilled water.

   b. 0.1M $NaH_2PO_4.2H_2O$ (MW=156.03) was prepared by dissolving 1.56g in 100ml of distilled water.

   Finally, 0.1M phosphate buffer was prepared by adding (a) to (b) and the pH adjusted to 7.4 with drops of concentrated HCl or NaOH as the case may be.

2. **100μmol/L Xylenol Orange**

   0.01g of xylene oxide (XO), (mol. Wt.=760.6) was dissolved in 200ml of distilled water.

3. **250μmol/L Ammonium Ferrous Sulphate (AFS)**

   0.02g of AFS, $(NH_4)_2SO_4.FeSO_4.6H_2O$, (mol. wt.=392.14) was dissolved in 200ml of distilled water.

4. **100mM/L Sorbitol**

   3.64g of sorbitol, $C_6H_{14}O_6$, (mol. wt.=182.2) was dissolved in 200ml Of distilled water.

5. **250mmol/L Sulphuric Acid**

   1ml of 1M H_2SO_4 was made up to 40ml with distilled water.

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Procedure

The assay mixture is thoroughly mixed by vortexing till it foamed. A pale pink colour complex is generated after incubating for 30 minutes at room temperature. The absorbance was read against a blank (distilled water) at 560nm wavelength.

3.5.2.7 DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY

The SOD activity was determined by the method of Misra and Fridovich, (1972).

Principle

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide (O_2^{-}) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O_2^{-} introduced increased with increasing pH (Valerino and McCormack, 1971) and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide (O_2^{-}) radical and hence inhibited by superoxide dismutase.

Reagents

1. **0.05M Carbonate buffer (pH 10.2)**

   3.58g of $Na_2CO_3.10H_2O$ and 1.05g of $NaHCO_3$ were dissolved in 200 ml of distilled water. The pH was adjusted to 10.2 and then made up to 250 ml with distilled water.

2. **0.3mM Adrenaline**

   0.01g of fresh adrenaline (epinephrine) was dissolved in 200ml distilled water.

Protocol

One ml of sample was diluted in 9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted sample was added to 2.5 ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed

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by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Calculation

$$\text{Increase in absorbance per minute} = \frac{A_3 - A_0}{2.5}$$

Where A_0= absorbance after 30 seconds

A_3= absorbance after 150 seconds

$$\%\ \text{inhibition} = 100 - 100 \times \frac{\text{increase in absorbance for substrate}}{\text{increase in absorbance for blank}}$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.

3.5.2.8 DETERMINATION OF CATALASE ACTIVITY

Catalase activity was determined according to the method of Sinha (1971).

Principle

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colorimetrically at 570-610nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

Reagents

1. **5% $K_2Cr_2O_7$ (Dichromate Solution)**

   5% $K_2Cr_2O_7$ (Hopkins and Williams, England) was dissolved in 80ml of distilled water and made up to 100ml with same.

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2. 0.2M H_2O_2 (Hydrogen Peroxide)

11.50ml of 30% (w/w) H_2O_2 were diluted with distilled water in a volumetric flask and the solution made up to 500ml.

3. Dichromate/acetic acid

This reagent was prepared by mixing 5% solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 by volume) and could be used indefinitely.

4. Phosphate Buffer (0.01M, pH 7.0)

3.58g of $Na_2HPO_4.12H_2O$ and 1.19g $NaH_2PO_4.2H_2O$ were dissolved in 900ml distilled water. The pH was adjusted to 7.0 and distilled water was added to make up to 1 litre.

Procedure

Colorimetric determination of H_2O_2

Different amounts of H_2O_2, ranging from 10 to 100 µmoles were taken in small test tubes and 2ml of dichromate/acetic acid were added to each. Addition of the reagent instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10 minutes in a boiling water bath changed the colour of the solution to stable green due to formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3ml and the optical density measured with a spectrophotometer at 570nm. The concentration of the standard curve were plotted against absorbance.

Determination of catalase activity of samples

0.2ml of supernatant fraction of the liver, kidney and testicular homogenate were mixed with 0.8ml distilled water to give a 2:10 dilution. The assay mixture contained 2ml of H_2O_2 solution (800µmoles) and 2.5ml of phosphate buffer, pH 7.0 in a 10ml flat bottom flask. 1ml of properly diluted sample was rapidly mixed with the reaction mixture by a gentle swirling motion at room temperature. 1ml portion of the reaction mixture was withdrawn and blown into 2ml dichromate/acetic acid reagent at 60 second interval. The hydrogen peroxide contents of the withdrawn sample were determined by the method described above.

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Calculation

Catalase activity was obtained by plotting the standard curve and the concentration of the remaining H_2O_2 was extrapolated from the curve.

H_2O_2 consumed = 800µmoles – H_2O_2 remaining

$$\text{Catalase activity} = \frac{H_2O_2 \text{ consumed}}{\text{Mg protein}}$$

3.5.3 Determination of Aspartate amino transferase (AST) activity in the serum

AST activity was determined following the principle described by Reitman and Frankel, (1957). One unit/L of AST is defined as the liberation of 1mmol of pyruvate per minute at 37°C incubation per litre of serum.

Principle

α-Oxoglutarate + L-aspartate —— AST ——→ L-glutamate + Oxaloacetate

Aspartate aminotransferase was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine.

Reagents

Solution 1

Phosphate buffer (100mmol/L, pH 7.4), L-aspartate (100 mmol/L) and α-oxoglutarate (2mmol/L)

Solution 2

2, 4-dinitrophenylhydrazine (2 mmol/L)

Solution 3

0.4 mol/L sodium hydroxide solution (NaOH)

Procedure

0.1ml of serum sample was mixed with solution 1 i.e. phosphate buffer (100mMol/L, pH 7.4), L-aspartate (100mMol/L) and α–oxoglutarate (2mMol/L) and the mixture incubated for exactly 30 minutes at 37°C. Then, 0.5ml of solution 2, i.e. 2,4-dinitrophenylhydrazine (2mMol/L) was added to the reaction mixture and allowed to stand for exactly 20 minutes at 25°C. Then 5.0ml of solution 3 i.e. NaOH (0.4mol/L) were added and the absorbance read against the reagent blank after 5 minutes at 546nm.

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Reagent blank was prepared as described above replacing sample with 0.1ml of distilled water.

3.5.4 Determination of Alanine amino transferase (ALT) activity in the serum

Principle:

ALT activity was determined according to the principle described by Reitman and Frankel (1957).

The enzyme alanine aminotransferase catalyse the transfer of amino group from L-alanine and α-oxoglutarate to form L-glutamate and oxaloacetate.

$$\alpha\text{-oxoglutarate} + \text{L-alanine} \xrightarrow{ALT} \text{L-pyruvate} + \text{oxaloacetate}$$

The oxaloacetate formed is unstable and is quantitatively decarboxylated to pyruvate which is then complexed with 2, 4-dinitrophenylhydrazine (DNPH) to produce an intensely coloured hydrazone on the addition of NaOH. This coloured complex absorbs light at 530-550nm. Thus alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine. One unit/L of ALT is defined as the liberation of ImMol of pyruvate per minute at 37°C incubation per litre of serum.

Reagents

Solution 1

Phosphate buffer (100mMol/L, pH 7.4), L-alanine (200mMol), and oxoglutarate (2mMol/L)

Solution 2

2, 4-dinitrophenylhydrazine (2mmol/L)

Solution 3

0.4 mol/L NaOH

Procedure

0.1ml of serum sample was mixed with solution 1 i.e. phosphate buffer (100mmol/L, pH 7.4), L-alanine (200mmol/L) and α-oxoglutarate (2.0mmol/L) and the mixture incubated for exactly 30 minutes at 37°C. Then, 0.5ml of solution 2 i.e. 2,4-dinitrophenylhydrazine (2.0mmol/L) was added to the reaction mixture and allowed to

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stand for exactly 20 minutes at 25°C. Then 5.0ml of solution 3 i.e. NaOH (0.4mol/L) were added and the absorbance read against the reagent blank after 5 minutes at 546nm.

Reagent blank was prepared as described above replacing sample with 0.1ml of distilled water.

3.5.5 Determination of activity of Alkaline Phosphatase (ALP).

Activity of ALP can be measured using various phosphate esters as substrates. The principle is that ALP hydrolyses p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate at alkaline pH. The nitrophenol formed shows an absorbance maximum at 405nm.

p-nitrophenyl phosphate + H_2O –ALP— phosphate + p-nitrophenol

Reagents

ALP Reagent A

This contained diethanolamine 1.214mol/litre in buffer pH 9.8 and 0.607nmol/liter of magnesium ions.

ALP Reagent B

This contained 60.8nmol of p-nitrophenyl phosphate in a litre of distilled water.

Procedure

This was as described by Sigma Diagnostics, (1991). The ALP reagents A and B were maintained at 30°C in a water bath after their constitution. To 2.7ml of the ALP reagent A was added 0.05ml of the serum sample and mixed immediately by inversion. This was incubated for 1 minute at 30°C. 0.25ml of the ALP reagent B was then added, mixed immediately and taking the initial absorbance at 405nm with water as reference.

The incubation was continued at 30°C and the absorbance taken again exactly after 1, 2 and 3 minutes following the initial absorbance readings.

Calculation

$$\text{ALP Activity (U/L)} = \frac{A \times Tv \times 1000}{18.45 \times LP \times Sv}$$

Where:

A = change in absorbance per minute at 405nm

Tv = Total volume (ml)

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Sv = Sample volume (ml)

18.45 = millimolar absorptivity of p-nitrophenol at 405nm

Lp = Light path (1cm)

1000 = Conversion of units per ml to units per litre

3.5.6 Assay for Urea

The method used for determining urea concentration in the serum of the rats was the Urease-Berthelot Colorimetric method. Assay kits obtained from Randox Laboratories in the United Kingdom were used for this test.

Principle:

Urea in serum is hydrolysed by urease to ammonia which is then measured photometrically by Berthelot's reaction.

Urea + H_2O --urease--→ $2NH_3 + CO_2$.

NH_3 + hypochlorite + phenol ——→ indophenol (blue compound)

Reagents

a) **Reagent 1**

EDTA (116mmol/l), Sodium nitroprusside (6mmol/l) and urease enzyme (1g/l) are mixed together gently and stored at 2 to 8 °C.

b) **Reagent 2**

Phenol (120mmol/l) is diluted with 660ml distilled water and stored in a dark bottle at 2 to 8°C.

c) **Reagent 3**

Sodium hypochlorite (27mmol/l) and 0.14M sodium hydroxide are diluted with 750ml distilled water and stored in a dark bottle at 2 to 8°C.

d) **Urea Standard Solution**

Procedure:

The reagents were pipetted into test tubes. The mixtures were incubated in the test tubes at 37°C for 10 minutes. 2.5ml of both Reagents 2 and 3 are then added to all the test tubes. The solutions are immediately mixed and incubated once again at 37°C for 15 minutes. The colour of the indophenol product formed is

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stable for at least 8 hours and thus absorbance readings are taken after the reaction is complete. The absorbance of the samples and the standard are read against the blank at 546nm. Urea concentration in the serum samples is then calculated as follows:

$$\text{Urea concentration} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Standard concentration (mg/dl)}$$

Where Urea Standard Concentration = 80mg/dl.

3.5.7 Assay for Creatinine

Creatinine is derived from creatine and creatine phosphate in muscle tissue and may be defined as a nitrogenous waste product. It is excreted from the body in the urine via the kidney.

Creatine is regarded as the most useful endogenous marker in the diagnosis and treatment of kidney disease because its plasma concentration is relatively independent of protein ingestion, water intake, rate of urine production and exercise. Thus, elevation of plasma creatine is indicative of under-excretion suggesting kidney impairment.

Assay kit obtained from Randox Laboratories, United Kingdom was also used for this test.

Principle

Creatinine in alkaline solution reacts with picrate to form a coloured complex.

Reagents

a) **Reagent Mixture**

This is made up of equal volumes of picric acid (35mmol/l) and sodium hydroxide (1.6mol/l) and stored at 15 to 25°C for a maximum of 5 hours.

b) **Creatinine Standard Solution (2mg/dl)**

c) **Trichloroacetic acid, TCA (1.2mol/l)**

Procedure

To deproteinize the serum samples, 1ml of TCA and 1ml of the serum samples are introduced into tubes. The resulting solutions are mixed well to evenly disperse the precipitate and then centrifuged at 2500rpm for 10 minutes. The supernatants are then obtained for analysis of the creatinine concentrations.

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The supernatant and other reagents are then pipetted into test tubes.

The resulting mixture (after following the protocol above) is allowed to stand at 25°C for 20 minutes. Absorbance readings are then taken at 520nm against the blank for the samples and the standard.

Creatinine concentration is calculated from the absorbance readings as follows:

$$\text{Creatinine Conc.} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 2\text{mg/dl}$$

3.5.8 Protocol for Semen Analysis

Sperm from the epididymis of freshly killed rats were released into the semen incubation medium. Semen incubation medium consisted of tris-(hydroxymethyl)-aminomethane (37.85g/l), citric acid anhydrous (21.15g/l) and D(-)fructose (10g/l) (Roca *et al.*, 2000). These samples were then placed in a water bath at 37°C. The motility and viability of the sperm samples were estimated at 0, 1, 2 and 3 hours by visual examination under low-power magnification using light microscope. Live and dead sperm were estimated using 0.5% eosin stain.

3.5.8.1 Sperm Motility Assay

A small drop of semen was placed on a slide and diluents (physiological saline and 2.9% sodium citrate buffer solution) were added drop wise until the desired dilution was obtained. Cover slips were put on the smear of the sodium citrate diluted semen and were used for the estimation of spermatozoa motility. Observation was done at x100 magnification.

3.5.8.2 Live-Dead Count or Examination of Sperm

Principle:

The live-dead staining principle is based upon the observation that eosin B penetrate and stain the dead sperm cells whereas the viable cell repel this stain. In order to benefit from the live-dead method, the staining should be done without delay. The live-dead count supplements rather than replaces the motility tests.

3.5.8.3 Testicular and Epididymal Sperm Number

Epididymal sperm was collected by chopping the epididymis in 5ml physiological saline. The epididymal sperm was counted by the method as described by

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Yokoi *et al.* (2003). Briefly, the epididymis was minced with anatomical scissors in 5ml of physiological saline, placed in a rocker for 10 minutes and incubated at room temperature for 2 minutes. The supernatant fluid 25mg eosin per 100ml of distilled water. 100µl of the diluted sperm suspension was transferred to each counting chamber of the heamocytometer and allowed to stand for 5 minutes for counting under a light microscope at x200 magnification. For testicular spermatid head counts, the testis was weighed following the removal of the tunica albugina, and the tissue was then homogenized. 1ml of the testis homogenate was removed and sonicated (Meistrich, 1993), and an aliquot was placed in a heamacytometer to count late spermatid nucleus and the data was expressed as the number of spermatid heads per testis.

3.5.8.4 Sperm Morphology and Percentage Viability Assay

After sacrifice, the rat testiscules were removed immediately and placed in well insulated ice box maintained at 0-4°C. The epididymis were trimmed off the body of the testes and semen samples collected from the caudal through an incision with scalpel blade into the lumen and using 2-4 drops of 2.9% buffered sodium citrate kept at body temperature as the fluid. Aliquot of sperm suspension were stained using Wells and Awa stain for morphological examination and 1% eosin B and 5% nigrosine in 3% sodium citrate dehydrate for live-dead ratio.

3.5.8.5 Determination of Daily Sperm Production

Daily sperm production was determined using three frozen left testes from control and treated rats according to Joyces, (1993). Briefly after the testes have being removed and weighed, they were homogenized for 3 minutes in 25ml of physiological saline containing 0.05% (v/v) Triton X-100. Sample aliquots of the 5.5µl were then placed on the haemocytometer and counted twice at x100 magnification under microscope to determine the average number of spermatids per sample. These values were used to obtain the total number of spermatids per testis and this was then divided by the testes weight to give spermatids per gram of testes. Developing spermatids spend 4.61 days in rat testes. Thus the values for the number of spermatids per testis were divided by 4.61 to obtain daily sperm production.

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3.5.8.6 Histology

For morphological studies, testes and epididymides were fixed in Bouin's solution for six hours, embedded in paraffin, and sectioned at 6μm. The sectioned were mounted on slides and stained with haemotoxylin-eosin according to Hotchkis and McManuus (1961).

3.5.8.7 Statistical Analysis

All the results were subjected to statistical analysis using Analysis of Variance (ANOVA) and Pearson Correlation Coefficient. Results were regarded significant at $P<0.05$. It is not significant when $P\geq0.05$.

3.6 EXPERIMENT SIX

3.6.1 Biochemical Characterization of the *Lablab purpureus* seed proteins

MATERIALS AND METHODS

The grain legumes used for this study include the three varieties of lablab beans. The source of the lablab beans has been indicated earlier.

3.6.2 Protein Extraction

100mg of dehulled lablab seeds were ground using sterile mortars and pestles and added into 1ml 0.02M borate buffer of pH 8.9 containing 0.5M sodium chloride. Extraction was done by keeping the suspension overnight at $4^{O}C$ in a refrigerator and centrifuging at 21,000g for 20 minutes (Pedalino *et al.*, 1990) and the protein in the supernatant was collected and separated on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

In the protocol as described by Leammeli, (1970), a stacking and separating gel preparation of 4%, 0.125M tris and 12%, 0.375M tris respectively was used. Samples including both the low and high range molecular markers were diluted at different ratios 1:3,1:4,1:5 and 1:6 (v/v) in loading buffer and run at a constant voltage of 150 for an hour after which the gel was stained in coomasie brilliant blue and the molecular weights of the protein sub-units determined after destaining for 2 hours by a calibration graph obtained using the molecular weight markers.

The following proteins were used as marker proteins:

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Table 7: Molecular Weight Markers

High range marker	Molecular weight (Daltons)
Myosin from rabbit muscle	205,000
B-Galactosidase from *E. coli*	116,000
Phosphorylase b from rabbit muscle	97,000
Albumin, bovine serum	66,000
Glutamic dehydrogenase from bovine liver	55,000
Ovalbumin from chicken egg	45,000
Glyceraldhyde-3-phosphate-dehydrogenises	36,000
Low range marker	**Molecular weight (Daltons)**
Carbonic anhydrase from bovine erythrocytes	29,000
Trypsinogen from bovine pancreas	24,000
Trypsin inhibitor from soyabean	20,000
Lactoglobulin	18,400
α- Lactnibumin from bovine milk	14,200
Aprotinin from bovine lung	6,500

Jaccard's similarity index to compare the electrophoretic patterns of the different grain legumes as a measure of pattern homology was calculated according to Sneath and Sokal (1973) using the formula:

$$\frac{\text{Number of bands in common}}{\text{Number of different bands + number of bands in commmon}}$$

The molecular weights of the proteins were read off a standard graph prepared by plotting the molecular weights of some standard proteins against their relative electrophoretic mobilities.

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CHAPTER FOUR

RESULTS

4.1 EXPERIMENT ONE

Determination of the Proximate Analysis and Mineral Elements in Lablab beans.

Introduction

The application of nutritional science to humans or to growing farm animals is founded upon volumes of literature containing chemical analyses of foods and feeds (Ologhobo, 1980). Many of the methods in use today for the analysis of foods are procedures based on a system introduced initially about one hundred (100) years ago by two German scientists, Henneberg and Stohmann for the analysis of animal feedstuffs and are described as the Proximate Analysis of Foods (James, 1996). This scheme of analysis involves the estimation of the main components of a food using procedures that allow a reasonably rapid and acceptable measurement of various food fractions without the need for sophisticated equipment or chemicals. Terms such as crude fat and crude protein are a reflection of the fact that the estimations made do not necessarily give a measure of the true value of the food fraction in question but are, however, adequate for most requirements of the food analyst, particularly in view of the fact that to obtain the true value might require procedures involving greater time and cost (James, 1996).

The accumulated wealth of information on nutrient composition of foods and feedstuffs for humans and animals must be continually updated (Ologhobo, 1980). Plant breeders have accepted improved nutritional composition as a valid breeding objective and are now releasing crop varieties, more resistant to pests and microbial infections and with improved compositional assay. These varieties must be tested chemically first and then biologically, for their overall nutritional characteristics in addition to the individual nutrient levels for which they are selected. The plant breeders also require a constant feedback on crops for improved nutrient composition for incorporation into their breeding objectives.

The move from traditional, classical or "wet chemistry" techniques to modern Instrumental methods has not necessarily meant that the traditional methods have been

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discontinued since, in many instances, instrumental methods require an initial calibration of the instrument against results produced by the traditional methods (James, 1996). The choice of methods used for the analysis of foods is dependent on a number of factors. Their desirability or otherwise needs to be considered in deciding on a particular analytical procedure. They are: (i) Precision, (ii) Reproducibility, (iii) Accuracy, (iv) Simplicity of operation, (v) Economy, (vi) Speed, (vii) Sensitivity, (viii) Specificity, (ix) Safety and (x) Official approval.

The eventual choice of a method will thus depend on which of the above factors is most critical. In matters of dispute or involving legislative requirements, the use of an officially approved method could be of utmost importance, while for the purposes of routine analysis for quality control, speed, cost and precision could have a more important bearing (James, 1996).

This study was done to give a knowledge of the proximate compositions of the three varieties of lablab beans (*Lablab purpureus*).

Procedure

The procedure is as reported under Methodology in section 3.1

Proximate Composition is shown in Table 8 below

Table 8: Proximate Composition of *L. purpureus* seeds varieties

Sample	%Crude Protein	%Crude Fat	%Crude Fibre	% Ash	% Nitrogen Free Extract	Gross Energy (Kcal/g)	%Dry Matter	% Moisture
Rongai Brown	* 24.15 ±0.23	9.74 ± 0.74	12.69 ± 0.12	*4.28 ±0.15	39.27 ± 0.40	*3.10 ±0.15	89.96 ±0.31	10.04 ±0.21
Rongai White	23.10 ±0.80	9.56 ±0.38	* 13.12 ±0.15	3.97 ±0.15	40.29 ±0.15	2.93 ±0.59	*90.04 ±0.40	9.96 ±0.04
Highworth Black	22.75 ±0.53	9.63 ±0.85	12.98 ±0.15	4.12 ±0.12	*40.39 ±0.38	3.00 ±0.26	89.87 ±0.19	*10.13 ±0.19
Mean ± SEM	23.33 ± 0.32	9.64 ± 0.65	12.93 ± 0.14	4.12 ± 0.14	39.93 ± 0.31	3.008 ±0.33	89.96 ± 0.33	10.04 ± 0.15

* Significant at $P<0.05$

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Rongai brown recorded the highest crude protein of 24.15% while Highworth black records the least having 22.75%. For crude fat, Rongai brown had the highest of 9.74% while Rongai white had the least of 9.56%. For crude fibre, Rongai white had the highest of 13.12% while Rongai brown had least of 12.69%. For the Ash content, Rongai brown had the highest of 4.28% while Rongai white had the least of 3.97%. Nitrogen Free extract shows that Highworth black had the highest value of 40.39% while Rongai brown had the least of 39.27%.

For Gross energy, Rongai brown had the highest having 3.10Kcal/g while Rongai white had the least of 2.93Kcal/g. Dry matter reveals that Rongai white had the highest of 90.04% while Highworth black had the least of 89.87%. The moisture content revealed that the Highworth black had the highest of 10.13% while the Rongai white had the least of 9.96%.

Mineral elements in *L. purpureus* seeds is shown in Table 9

Table 9 Mineral Elements in *L. purpureus* seeds

Sample	Mg%	Ca%	Na%	K%	Mn%	P%	Fe (ppm)
Rongai Brown	0.34± 0.02	0.67± 0.01	0.18± 0.01	1.53± 0.01	0.08± 0.01	0.48± 0.01	157.0± 1.01
Rongai White	0.34± 0.02	0.67± 0.01	0.18± 0.01	1.53± 0.01	0.08± 0.01	0.48± 0.01	157.0± 1.01
Highworth Black	0.34± 0.01	*0.71± 0.01	0.17± 0.01	*1.59± 0.03	0.05± 0.01	*0.55± 0.07	* 168.0± 0.01
Mean± SEM	0.34± 0.01	0.70± 0.01	0.17± 0.00	1.57± 0.02	0.07± 0.01	0.50± 0.03	160.00± 2.71

* Significant at $P<0.05$

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The magnesium content recorded 0.34% for all the three varieties of lablab beans. The calcium contents of the raw lablab seeds ranged between 0.67% in Rongai brown and Rongai white to 0.71% in Highworth black. The sodium content ranged from 0.17% in Highworth black to 0.18% in Rongai brown and Rongai white. The most abundant element in all the three varieties of lablab seeds is potassium, ranging from 1.59% in Highworth black to 1.53% in Rongai brown and Rongai white. Manganese content ranged from 0.05% in Highworth black to 0.08% for both Rongai brown and Rongai white. The phosphorus content ranged from 0.48% in Rongai Brown and Rongai white to 0.55% in Highworth black. The concentration of iron ranged from 157 parts per million (ppm) in Rongai brown and Rongai white to 168 ppm in Highworth black.

Conclusion.

This study reveals that *Lablab purpureus* has some qualities and potentials above other common legumes like the cowpea, soyabean and pigeonpea, as manifested in its high crude fibre and crude protein content. Efforts should therefore be devoted to conducting more research to extend both technical and practical knowledge about *Lablab purpureus* so that its full potential may be achieved. This legume should be exploited as food supplements because of their potentials as rich protein sources.

4.2 EXPERIMENT TWO

Quantification of the Anti-nutritional Factors and Phytochemical Analyses of the Lablab beans.

Introduction

A major factor limiting the wider use of many tropical plants is the ubiquitous occurrence in them of a diverse range of natural compounds capable of precipitating deleterious effects in man and animals (Osagie, 1998; Pariza, 1996). Manifestations of toxicity range from severe reduction in food intake and nutrient utilization to profound weight loss, neurological effects, and even death.

Compounds which act to reduce nutrient utilization and or food intake are often referred to as anti-nutritional factors (ANF). Legumes and other plant foods contain significant amounts of toxic or antinutritional substances, although legumes are a

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particularly rich source of these natural toxicants including protease inhibitors, saponins, haemagglutinins, tannins, cyanogens etc (Pariza, 1996). Although toxic compounds are widely distributed in the plant kingdom it is generally considered that tropical legumes contain a more complex array of these substances than other crop species. Some of these substances reduce the nutritional value of foods by interfering with mineral bioavailability and digestibility of proteins and carbohydrotes. The toxic factors may occur in all parts of the plant but the seed is normally the most conceotrated source. Consequently, most legume grains are highly toxic to animals if fed without adequate processing. The levels of deleterious substances in tropical legumes vary with plant specie and cultivar (Osagie, 1998). Knowledge of toxic substances naturally present in plants that are or may be used as food are necessary so as to be able to know the possible side effects of consuming plants that contain them either by improper processing or ignorantly as in field conditions in cases of grazing animals.

Procedure

The procedure is as reported under Methodology in section 3.2

The result of the Quantification of the Antinutritional Factors is shown in table 10 below.

Table 10. Quantification of the Antinutritional Factors

Sample	TIU/mg protein	HU/mg	Cynogenic Glycosides mg/kg	Oxalates mg/g	Phytates mg/g	Tannin mg/g	Saponain mg/g	Alkaloids mg/g
Rongai Brown	*44.8 ±0.57	23.7 ±0.10	185.0 ±0.89	9.3 ±0.36	13.6 ±0.27	*4.7 ±0.06	11.3 ±0.17	4.8 ±0.12
Rongai White	31.6 ±0.21	18.7 ±0.17	175.0 ±0.00	8.2 ±0.12	14.4 ±0.06	3.5 ±0.84	11.6 ±0.06	3.7 ±0.15
Highworth Black	39.5 ±0.29	*28.6 ±0.06	*195.0 ±0.57	*9.8 ±0.17	14.0 ±0.00	4.2 ±0.81	*12.1 ±0.06	*6.8 ±0.15
Mean ± SEM	38.6 ±0.36	23.7 ±0.11	185 ±0.49	9.1 ±0.22	14.0 ±0.11	4.1 ±0.57	11.7 ±0.09	5.1 ±0.14

* Significant at $P<0.05$

Trypsin inhibitor unit revealed that Rongai brown had the highest value of 44.8±0.57tiu/mg protein while Rongai white had the least value of 31.6±0.21tiu/mg.

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Values of Haemagglutinin revealed that Highworth black had the highest value of 28.6±0.06HU/mg while Rongai white had the least value of 18.7±0.171HU/mg. For cyanogenic glycosides, Highworth black had the highest value of 195.0±0.57mg/g while Rongai white had the least value of 175.00±0.00mg/g. Oxalate content shows that Highworth black had the highest value of 9.8±0.17mg/g while Rongai white had the least value 8.2±0.12mg/g. Phytate content shows that Rongai white had the highest value of 14.4± 0.06mg/g while Rongai brown had the least value of 13.6±0.27mg/g.

Tannin content shows that Rongai brown had the highest value of 4.7±0.06mg/g while Rongai white had the least value of 3.5±0.84mg/g. Saponin content shows that Highworth black had the highest value 12.1±0.06mg/g while Rongai brown had the least value of 11.3±0.17mg/g. Alkaloids content revealed that Highworth black had the highest value of 5.1±0.14mg/g while Rongai white had the least value of 3.7±0.15mg/g.

4.2.1 Phytochemical Screening of *Lablab purpureus* seeds

Introduction

Phytochemicals are plant chemicals or more appropriately defined as bioactive non-nutrient plant compounds in plant foods that have been linked to reduce the risks of major chronic diseases and cancers (Jing *et al.*, 2009). Phytochemicals in plants can have complementary and overlapping mechanisms of action, including gene expression in cell proliferation, cell differentiation, oncogenes and tumour suppressor genes, induction of cell-cycle arrest and apoptosis, modulation of enzyme activities in detoxification, oxidation and reduction, stimulation of the immune system, regulation of hormone metabolism, as well as antibacterial and antiviral effects (Sun *et al.*, 2002).

These natural compounds in plants belong to different molecular families which have various properties to human and animals. Some researchers are studying the activities of these molecules with a view to determine their use in clinical medicine. (Gupta and Kohli, 2010). The identification of these compounds in plants are of interest because of their beneficial effects and this study was therefore designed for this purpose.

Procedure:

The procedure is as reported under Methodology in section 3.2.2.

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PHYTOCHEMICAL ANALYSES is shown in Table 11 below

Table 11 Phytochemical Analysis of *L. purpureus* seeds

Analysis	Rongai brown	Rongai white	Highworth black
Alkaloid	Positive (++)	Positive (++)	Positive (++)
Cardenolides	Positive (++)	Positive (++)	Positive (++)
Anthraquinones	Negative (-)	Negative (-)	Negative (-)
Saponins	Positive (+++)	Positive (+++)	Positive (+++)
Tannins	Positive (+++)	Positive(+++)	Positive(+++)

Phytochemical analysis shows that alkaloids, cardenolides, saponins and tannins were present in all the three varieties of lablab beans.

EXPERIMENT THREE

4.3 Nutritional Studies of Lablab beans as affected by the inherent Anti-nutritional factors.

Introduction

Legumes for example, cowpea, soybean, pigeonpea and limabean can be considered to be very important in the Nigerian diets because they are grown and used as staple food commodity (Ologhobo, 1980). Because the principal elements in the Nigerian diet makes its nutritional quality undesirable, legumes come in as the most important source of protein. Accurate analysis of the nutrients contained in them, is a prerequisite for their most effective utilization and a basis for their nutritional enhancement. Abeke *et al.*, (2003) reported that most farmers would prefer to feed raw lablab beans to chicken in order to eliminate the cost of transporting the raw beans to the processor and also to eliminate other handling costs like heating. Little information is available in this area, especially with regards to different varieties of lablab beans (*Lablab purpureus*), a newly introduced legume to Nigeria, cultivated for food/feeds in humans and animals.

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It has been realized that the "available" as opposed to the total amino acids of a feeding stuff is more important in determining its value in a diet (Ologhobo, 1980). This has led to a series of investigations on methods of determining the available as opposed to the total amino acids in protein. This present study was designed to supply additional biological information on the three varieties of lablab beans (Rongai brown, Rongai white and Highworth black) and to attempt to establish the effects of the inherent antinutritional factors in them as they existed in the raw state. The main criteria for judgement are (i) change in weight (ii) feed intake, (iii) protein efficiency ratio, (iv) net protein ratio, (v) protein retention efficiency, (vi) feed conversion efficiency and (vii) protein intake.

Procedure

Twenty five weanling rats of Wistar strain obtained from the Animal House of the Faculty of Veterinary Medicine, University of Ibadan divided into 5 groups were used. The rats weighed 68g to 80g, the cages were numbered and the rats were housed individually in metabolic cages with a wire screen bottom located at the Department of Animal Science, University of Ibadan. The cages were provided with adequate devices for collection of faeces and urine of the rats they housed. The compositionof the standard reference diet and basal protein-free diet is shown in Table 4 above.

The feed was placed into small pots designed to minimize scattering of the feed. Fine wire screens were placed in the plastic trays below the cages to collect the faeces and spilled over feed, and to allow the urine to drip on to the tray below and be finally collected in a small urine cup. The spilled feed and faeces were separated by hand picking which enabled the separation of the particles of hair from the spilled feed and faeces. The animals were divided on the basis of their bodyweights into five groups of five rats each, such that the mean initial weights of each group were identical. The first three groups received the three varieties of the raw lablab beans while the fourth group received a reference protein diet for comparison, and served as control and the fifth group received a basal protein-free diet. All the diets were served to the rats in ground form after proper mixing. The rats were fed 10g experimental diets per day and feeding was done for 7 days. After a 3-days period, faecal samples were collected quantitatively

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daily for 4 days, bulked for each rat, weighed, dried at 105°C for 24 hours, ground into powder and stored according to the method of Pastuszewska *et al*,(2003) and of Agbede and Aletor,(2004). Duplicate samples of faeces and diets were taken for nitrogen determination by the Kjedahl method (AOAC, 1995). The feed consumed daily was obtained by weighing the feed given and subtracting from it the weight of whatever feed remained in the dish together with the spilled over feed after 24 hours. The weights of the rats were obtained at the start of the experiment and on the seventh day.

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Results of the Nutritional Studies on *L. purpureus* Seeds is shown in Table 12 below.

Table 12: Protein Quality Studies on *L. purpureus* Seeds

Sample	Initial BW (g)	Final BW (g)	Change in weight (g)	Feed Intake (g)/week	PER %	NPR %	PRE %	FCE %	Protein Intake (g)
Control	70.6± 2.61	87.0± 3.08 (18.90)	16.4± 2.70	39.44± 3.52	1.02± 0.22	5.40± 0.38	86.18± 5.80	41.8± 8.98	1617± 144.00
Rongai Brown	75± 5.0	*48.4± 5.46 (35.50)	*26.6± 1.52	*19.1± 1.24 (51.57)	*0.10± 0.00 (90.20)	*0.11± 0.01 (97.96)	*1.70± 0.14 (98.03)	*1.40± 0.13 (96.65)	*461.4± 29.90 (69.16)
Rongai White	71.0± 2.65	*42.0± 4.64 (40.85)	*29.0± 2.65	*23.5± 2.60 (40.42)	*0.09± 0.03 (91.18)	*0.10± 0.00 (98.15)	*1.60± 0.00 (98.14)	*1.30± 0.17 (96.89)	*513± 60.24 (66.42)
Highworth Black	74.0± 4.18	*49.0± 6.52 (33.80)	*25.0± 5.00	*23.2± 1.25 (41.18)	*0.08± 0.03 (92.16)	*0.10± 0.00 (98.15)	*1.64± 0.09 (98.10)	*1.10± 0.19 (97.37)	*528± 28.49 (67.35)

* Significant at $p<0.05$ ( ) % Difference

Key:

PER: Protein Efficiency Ratio

NPR: Net Protein Ratio

PRE: Protein retention Efficiency

FCE: Feed Conversion Efficiency

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All the rats fed the three varieties of lablab beans recorded significant decreases in final body weights as compared to the control rats with the rats fed the Rongai white giving the highest body weight decrease of 29.0g. For the feed intake, all the rats fed the lablab beans recorded decreases, with rats fed on Rongai brown having the least feed intake of 19.1g/week. For the PER, all the rats fed on the lablab beans also produced significant decreases as compared to control rats with rats fed the Higworth black giving the least value of 0.08%. For the NPR, all the rats fed the lablab beans also produced significant decreases with rats fed Rongai white and Highworth black producing the least value of 0.10%. For the PRE, all the rats fed the lablab beans also produced significant reductions as compared with control rats with the rats fed the Rongai white producing the least value of 1.60%. For the FCE, all the rats fed the lablab beans recorded significant decreases with rats fed Highworth black recording the least value of 1.10%. Alll the rats fed the three varieties of lablab beans produced significant reductions in protein intake as compared with control rats with the rats fed the Rongai brown variety producing the least protein intake of 461.4g.

The results of protein quality studies shown above reveal that there were significant decreases ($P<0.05$) in the final body weight, feed intake, protein efficiency ratio (PER), net protein ratio (NPR), protein retention efficiency (PRE), feed conversion efficiency (FCE) and protein intake in the rats fed the three varieties of lablab beans compared with the rats fed the control diet.

Conclusion

The significant reduction in the various parameters used for the nutritional evaluation of these three varieties of lablab beans could be attributed to the presence of the antinutritional factors inherent in them which reduces the nutrient intake and utilization by the rats.

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EXPERIMENT FOUR

4.4 Toxicological effects of the *Lablab purpureus* seeds

Introduction

The discovery that some of the common plants consumed by man and animals as food and concoction possesss deleterious effects appears to be growing in recent times (Oyeyemi *et al.*, 2007).

Although members of the Leguminosae enjoy widespread use as protein sources in human and animal nutrition, it has long been established that ingestion of these plants in the raw state produces various biochemical and physiological responses, usually accompanied by growth inhibition. The depression in growth is believed to be a consequence of inefficient utilization of dietary nutrients (Brambila *et al.*, 1978; Santidrian, 1981) caused by the presence of antinutritional factors in kidneybean, such as trypsin inhibitors, cyanogenic glycosides, haemagglutinnins (lectin), saponins and tannins (Eggum, 1980; Liener, 1980). However, the actual mode of action of these antinutritional factors remain largely unclear.

The effects of many poisonous substances in humans and animals often manifest in the production of typical clinical symptoms and gross and histopathologic lesions in the tissues. The lesions of poisoning are reported to be rarely characteristic. Nevertheless, the findings at autopsy are reported to provide definite clues to the nature of the poison (Clarke and Clarke, 1975). A valuable adjunct to the clinical diagnosis of poisoning is the determination of serum enzymes, which involves the detection of enzymes in the serum or plasma which are normally confined to the tissues and whose activities or concentrations are normally low in the serum or plasma (Schmidt and Schmidt, 1967). The poisons, when in sufficient concentration kill the tissue which they come in contact with or if the action is milder, they injure the tissues and initiate an acute inflammatory reaction. In toxicological studies, therefore, the most important evidence of toxicity, will be the observation of clinical symptoms and gross pathologic lesions in the tissues of the affected animals. Escape of enzymes as a result of the disruption of the hepatic parenchyma leads to necrosis and altered membrane permeability. This consequently affects the concentration of certain liver enzymes

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(Kaneko, 1980). The level of tissue damage can then be further evaluated histopathologically.

While the toxicological effects of most other legumes have been very widely studied, biochemical and pathological effects of lablab beans (*Lablab purpureus*) anti-nutritional factors have not been well documented. This study was therefore carried out to evaluate the biochemical and pathological effects of the lablab beans in the rat.

Procedure

Twenty weanling male albino rats of the Wistar strain obtained from the rat colony of the Department of Physiology, College of Medicine, University of Ibadan, were used for this study.

The rats with average body-weights of about 130-180g were randomly selected and housed in stainless-steel individual metabolic cages (Associated Crate Ltd, England) located at the Animal House of the Department of Animal Science, University of Ibadan. Five rats were put on each test diet. The experimental diets contained raw lablab beans; rongai white, rongai brown, and highworth black varieties obtained from RCMD unit of IITA, Ibadan. The rats in the control group were fed rat pellets manufactured by Bendel Feeds and Flour Mills Ltd, Edo State. The composition of the control feed is reported in Table 5 under section 3.4.1.

The feed was placed in small bowls firmly attached to the cages to minimize spillage and scattering. The spilled feed was collected in the containers placed under the individual cages. The experimental animals were fed with the normal rat diet for seven days and given water *ad libitum* to allow for acclimatization of the rats. The experiment lasted seven days during which 10g of feed were given daily but water was offered ad libitum. The feed were weighed using mettler electronic balance (Metler PM 4000).

Daily feed consumption was computed by collecting spilled feed and weighing it along with the feed remaining in the bowl. This was subtracted from 10g to give the actual daily feed consumed. Water was offered in well washed plastic drinking bottles with changes of water made every other day to prevent bacterial and fungal growth. Faecal output and texture were visually examined as excess pellets passed per animal per cage.

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Table 13: Haematological Parameters of rats fed with *L. purpureus* Seeds

Sample	PCV (%)	RBC ($x10^{12}$/L)	Hb (g/dL)	MCV (fl)	MCH (pg)	MCHC (g/dL)	WBC ($x10^9$/L)	Lymph ($x10^9$/L)	Neut ($x10^9$/L)	Mon ($x10^3$/μL)
Control	43.6± 1.34	9.36± 0.65	13.68± 0.53	46.75± 3.32	14.65± 0.70	31.50± 1.70	16.80± 1.05	10.28± 0.87	6.44± 0.30	0.00± 0.00
Rongai Brown	•30.40± 1.52 (30.28)	•5.02± 0.35 (46.37)	•9.0± 0.74 (34.21)	•60.78± 5.07 (23.08)	•17.96± 1.37 (18.43)	•29.70± 3.35 (5.71)	•8.78± 0.62 (47.74)	•5.68± 0.74 (44.75)	•3.16± 0.54 (50.93)	•0.06± 0.05 (100)
Rongai White	•35.4± 1.52 (18.81)	•6.46± 0.36 (30.98)	•11.60± 0.54 (15.20)	•54.90± 3.11 (14.85)	•18.03± 1.77 (18.75)	•32.82± 2.14 (4.02)	•12.56± 0.44 (25.24)	•8.34± 0.21 (18.87)	•4.30± 0.49 (33.23)	0.00± 0.00 (0)
Highworth Black	•32.4± 1.82 (25.69)	•5.76± 0.18 (38.46)	•10.70± 0.24 (21.78)	•56.24± 2.40 (16.87)	•18.60± 0.62 (21.24)	•33.11± 2.10 (4.86)	•10.78± 0.30 (35.83)	•7.68± 0.24 (25.29)	•3.43± 0.15 (46.74)	0.02± 0.04 (100)

• Significant at $P<0.05$

PCV: Packed Cell Volume

RBC: Red Blood Cell

Hb: Haemoglobin Concentration

MCV: Mean Corpuscular Volume

MCH: Mean Corpuscular Haemoglobin

MCHC: Mean Corpuscular Haemoglobin Concentration

WBC: White Blood Cell

Lymph.: Lymphocytes

Neut.: Neutrophils

Mon.: Monocytes

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All the rats fed with the three varieties of lablab beans recorded significant decreases in the level of PCV, RBC and Hb concentrations. The rats fed with the Rongai brown variety produced the least values of PCV (30.40%), RBC (5.02×10^{12}/L) and Hb (9.0g/dL). All the rats fed with the lablab beans recorded significant increases in the level of MCV. The rats fed with the Rongai brown variety had the highest value of MCV (60.78fl). All the rats fed with the lablab beans also recorded significant increases in the level of MCH. The rats fed with Rongai white and Highworth black varieties produced the highest values of MCH (18.03pg) and (18.60pg) respectively. For the MCHC, only the rats fed with the Rongai brown variety produced significant decrease of 29.70g/dL, while the rats fed the other varieties recorded significant increases in MCHC. For the levels of WBC, circulating lymphocytes and neutrophils, all the rats fed with the lablab beans recorded significant reductions. The rats fed with Rongai brown variety produced the least values of WBC (8.78×10^{9}/L), lymphocytes (5.68×10^{9}/L) and neutrophils (3.16×10^{9}/L).

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Table 14: Serum Biochemical Parameters of rats fed with *L. purpureus* Seeds

Sample	ALP (U/L)	AST (U/L)	ALT (U/L)	GGT (unit/mg protein)	Urea (mg/dL)	Creat (mg/dL)	Total Protein (mg/dL)	Alb. (mg/dL)	Glob. (mg/dL)	Initial. B.W (g)	Final B.W. (g)
Control	77.00± 2.65	94.70± 11.85	24.25± 3.86	10.50± 1.00	34.75± 7.5	0.45± 0.06	80.75± 2.99	47.8± 2.06	33.5± 4.0.1	137± 4.50	181.60± 4.63 (32.55)
Rongai Brown	*341.75± 35.42 (77.47)	*266.50± 21.69 (64.47)	*74.50± 4.73 (67.45)	*29.8± 2.95 (64.77)	*89.00 ± 5.00 (60.96)	*1.14± 0.05 (60.53)	*43.8± 2.68 (45.76)	*28.4±1 .67 (40.52)	*15.4±2. 19 (54.03)	168±8. 37	*128± 13.03 (23.81)
Rongai White	*155.00± 2.94 (50.32)	*173.50± 11.12 (45.42)	*40.75± 3.5 (40.49)	*13.8± 1.64 (23.91)	*45.67 ± 1.53 (23.91)	*0.53± 0.05 (15.09)	*54.0± 3.16 (33.13)	*25.6±2 .61 (46.39)	*28.4±2. 61 (15.22)	175± 5.0	*125± 20.62 (28.57)
Highworth Black	*177.00± 6.38 (56.50)	*235.50± 10.08 (59.79)	*53.75± 1.89 (54.88)	*21.5± 0.60 (51.16)	*65.50 ± 3.87 (46.95)	*0.83± 0.05 (45.78)	*53.25 ±2.75 (34.06)	*33.5±1 .91 (29.84)	*19.8±1. 71 (41.04)	160± 7.07	*124± 18.20 (22.50)

* Significant at $P < 0.05$

ALP: Alkaline hosphatase

AST: Aspartate Aminotransferase

ALT: Alanine Aminotransferase

GGT: Gamma-Glutamine Transferase

Creat.: Creatinine

Alb.: Albumin

Glob.: Globulin

BW: Body weight

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All the rats fed with the three varieties of lablab beans recorded significant increases in the values of ALP, AST, ALT, GGT, Urea and Creatinine. The rats fed the Rongai brown variety recorded the highest values of ALP (341.75U/L), AST (266.50U/L), ALT (74.50U/L), GGT (29.8 unit/mgprotein), Urea (89.00mg/dL), and Creatinine (1.14mg/dL). There were significant reductions in the concentration of total protein, albumin and globulin in all the rats fed with the lablab beans. The rats fed the Rongai brown recorded the least value for total protein (43.8mg/dL), Rongai white rats recorded the least value for albumin (25.6mg/dL) and Rongai brown rats recorded the least value for globulin (15.4mg/dL).

4.4.1 Histopathology

LIVER

Rongai Brown: Severe diffuse necrosis of hepatocytes (Plate 1)

Rongai White: Mild congestion of hepatic blood vessels (Plate 2)

Highworth Black: Mild congestion of hepatic blood vessels (Plate 3)

Control: No significant lesions were found (Plate 4)

KIDNEY

Rongai Brown: Moderate congestion of blood vessels (Plate 5)

Rongai White: Moderate vascular congestion and focal haemorrhages (Plate 6)

Highworth Black: Moderate congestion and marked tubular degeneration with presence of protein (Plate 7)

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Control: No significant lesions were found (Plate 8)

TESTES

Rongai Brown: Degeneration and necrosis of germinal epithelium (Plate 9)

Rongai White: Mild diffuse degeneration and necrosis of germinal epithelium (Plate 10)

Highworth Black: Mild degeneration of seminiferous epithelial cells with very few cells present in the tubules (Plate 11)

Control: No significant lesions were found (Plate 12)

RB= Rongai Brown; RW= Rongai White; HB= Highworth Black

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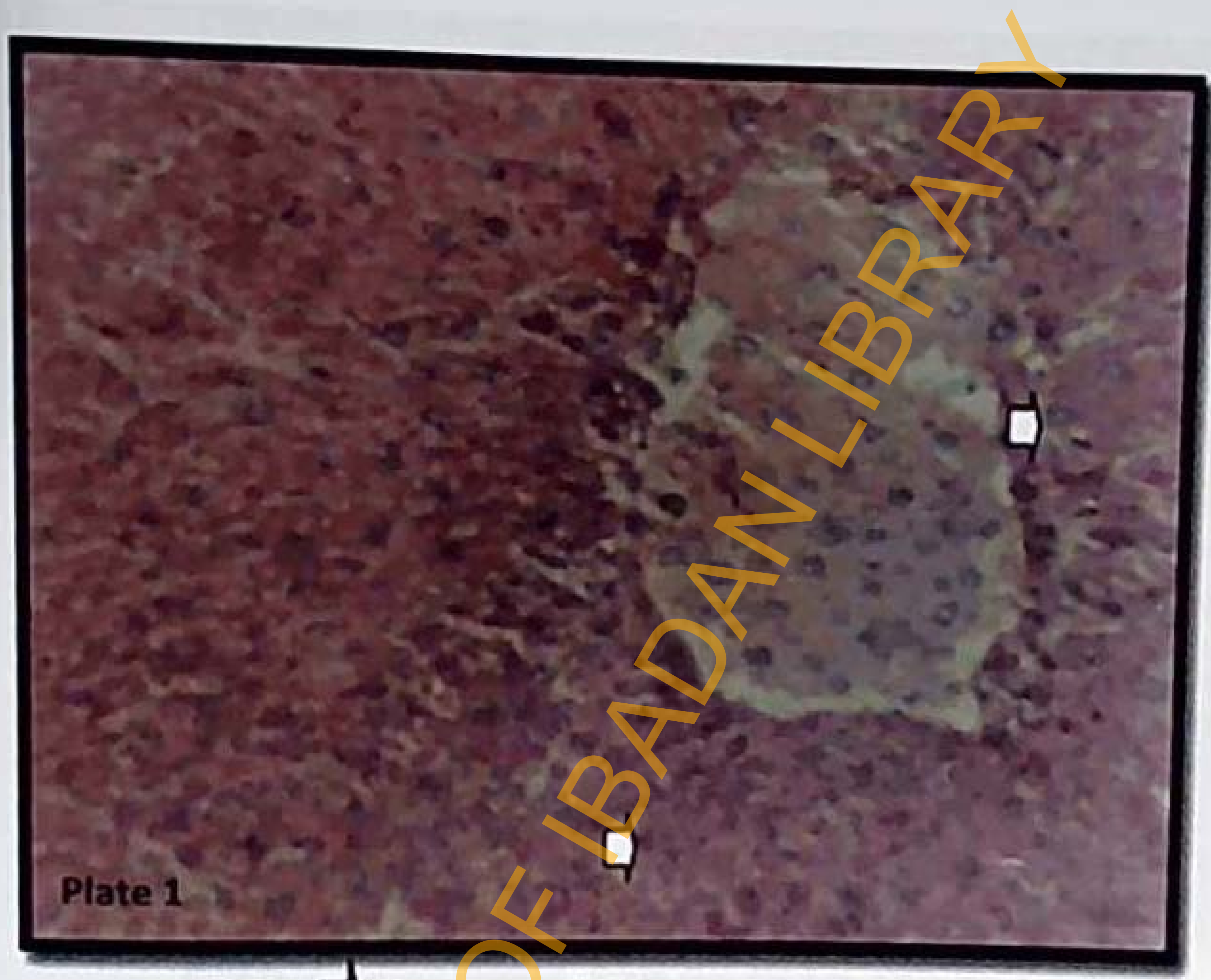

RB: Severe diffuse necrosis of hepatocytes

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RW: ⇨ Mild congestion of hepatic blood vessels

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HB: ⇨ Mild congestion of hepatic vessels.

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Control: No significant lesions were found.

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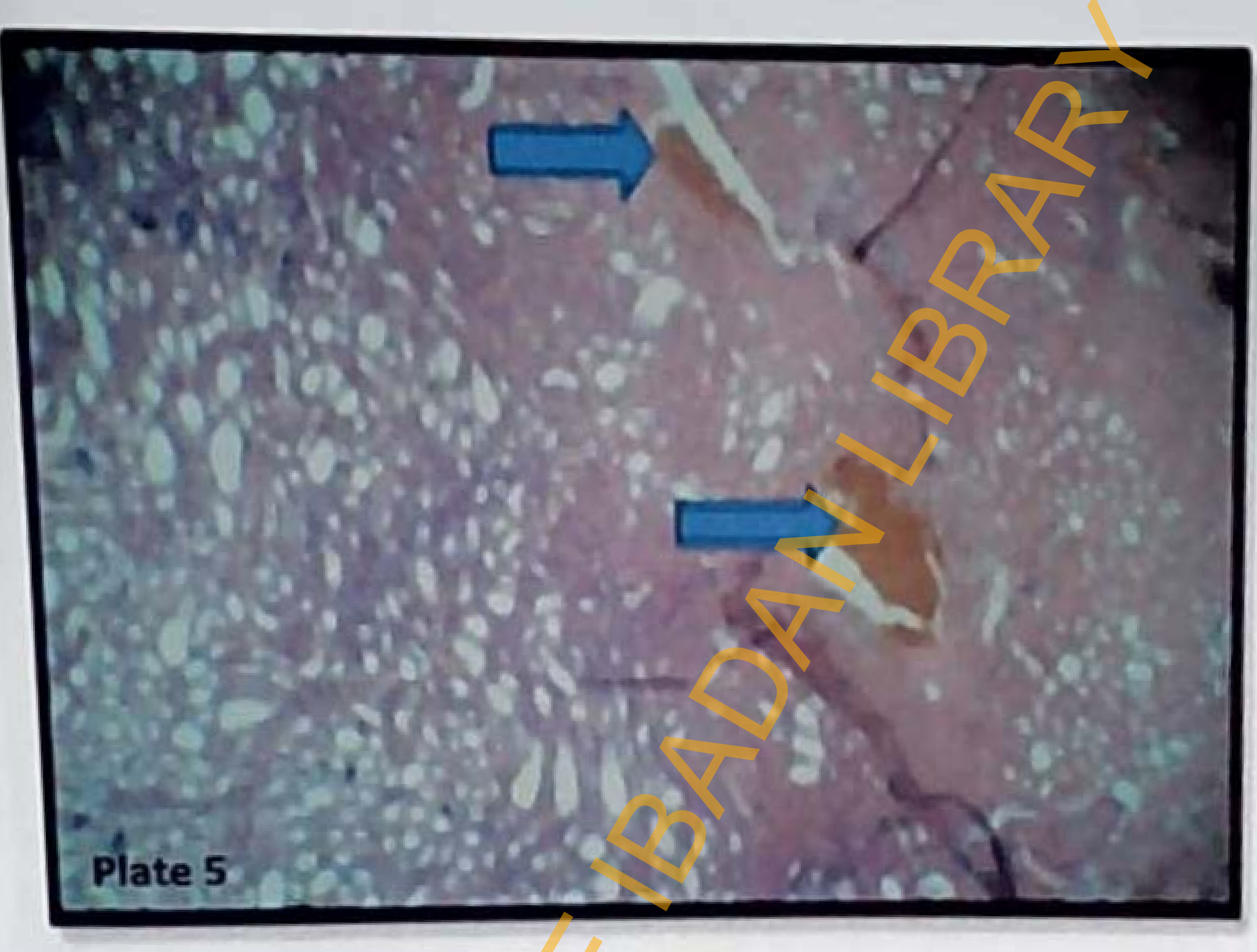

RB: ➡ Moderate congestion of blood vessels

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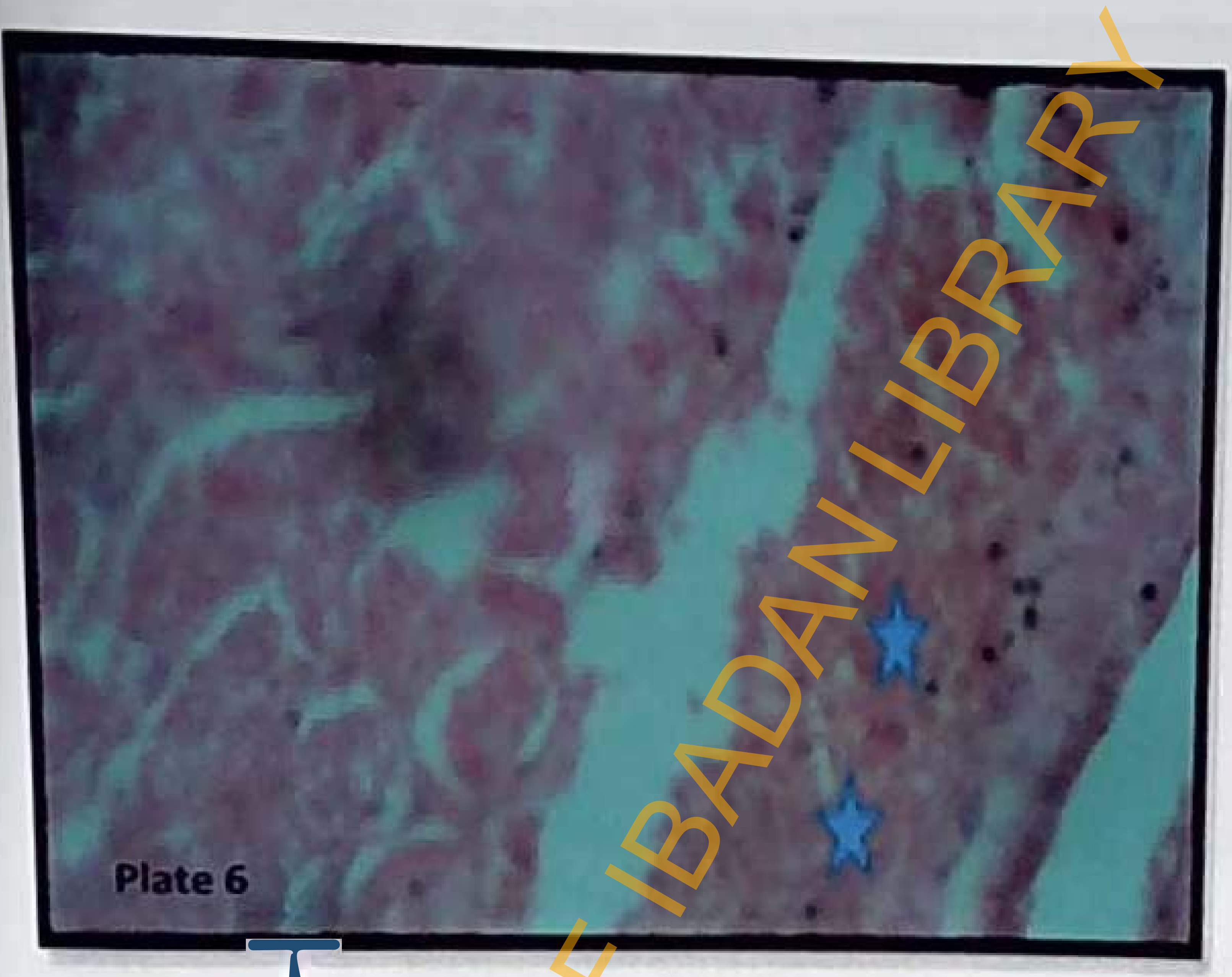

RW: Moderate vascular congestion and focal haemorrhages

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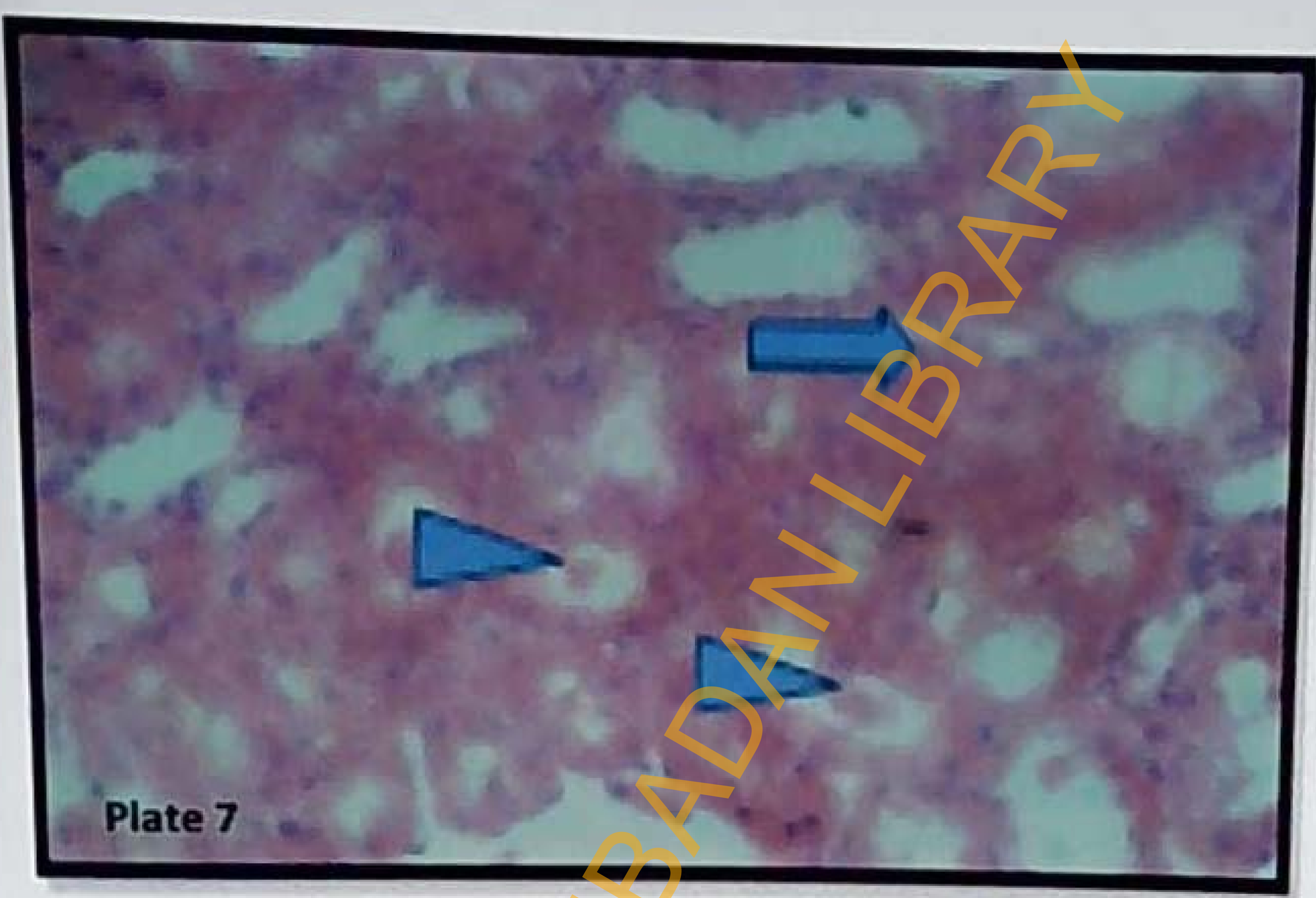

HB: ➡ Moderate congestion ▶ marked tubular degeneration with presence of protein

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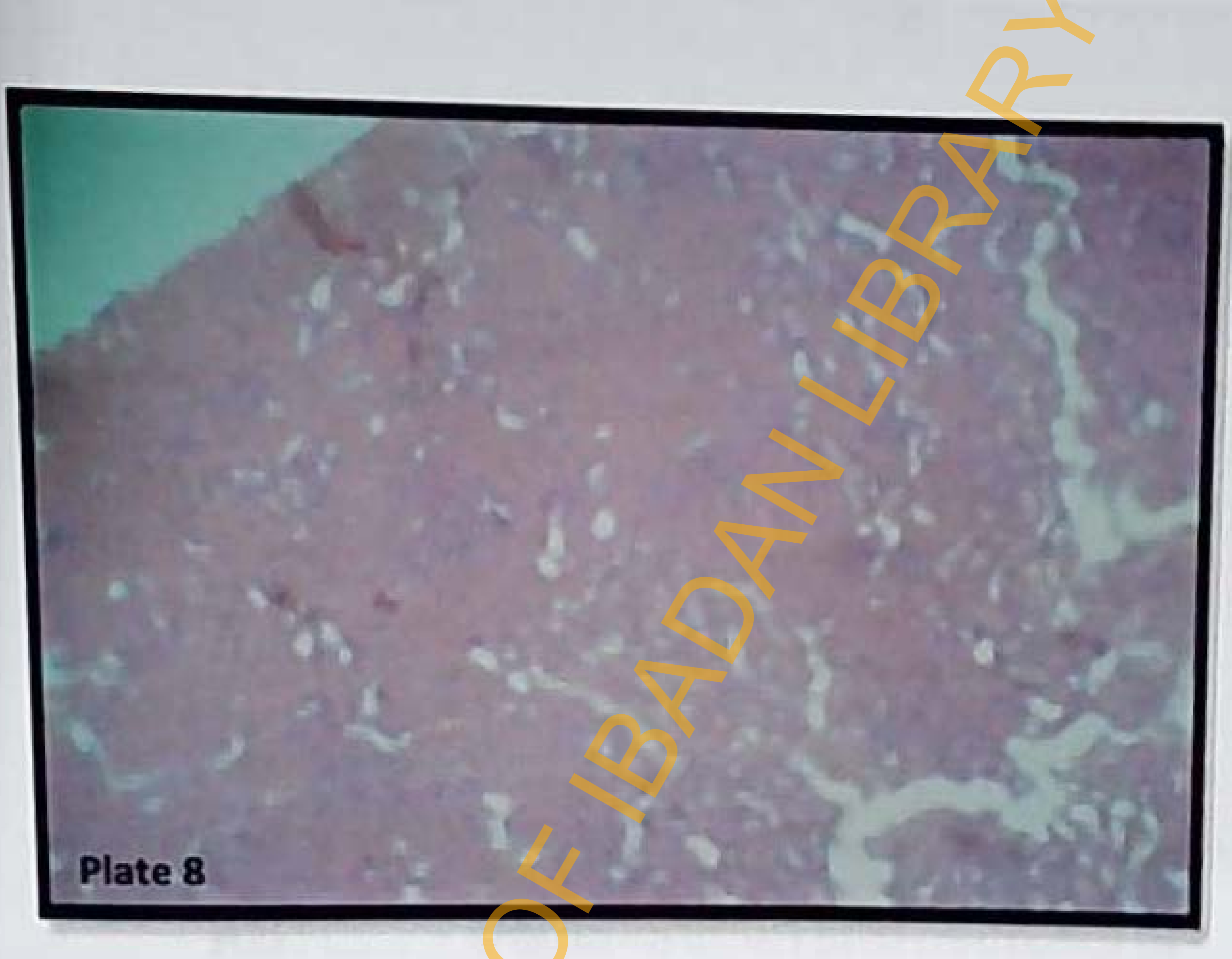

Control: No significant lesions were found

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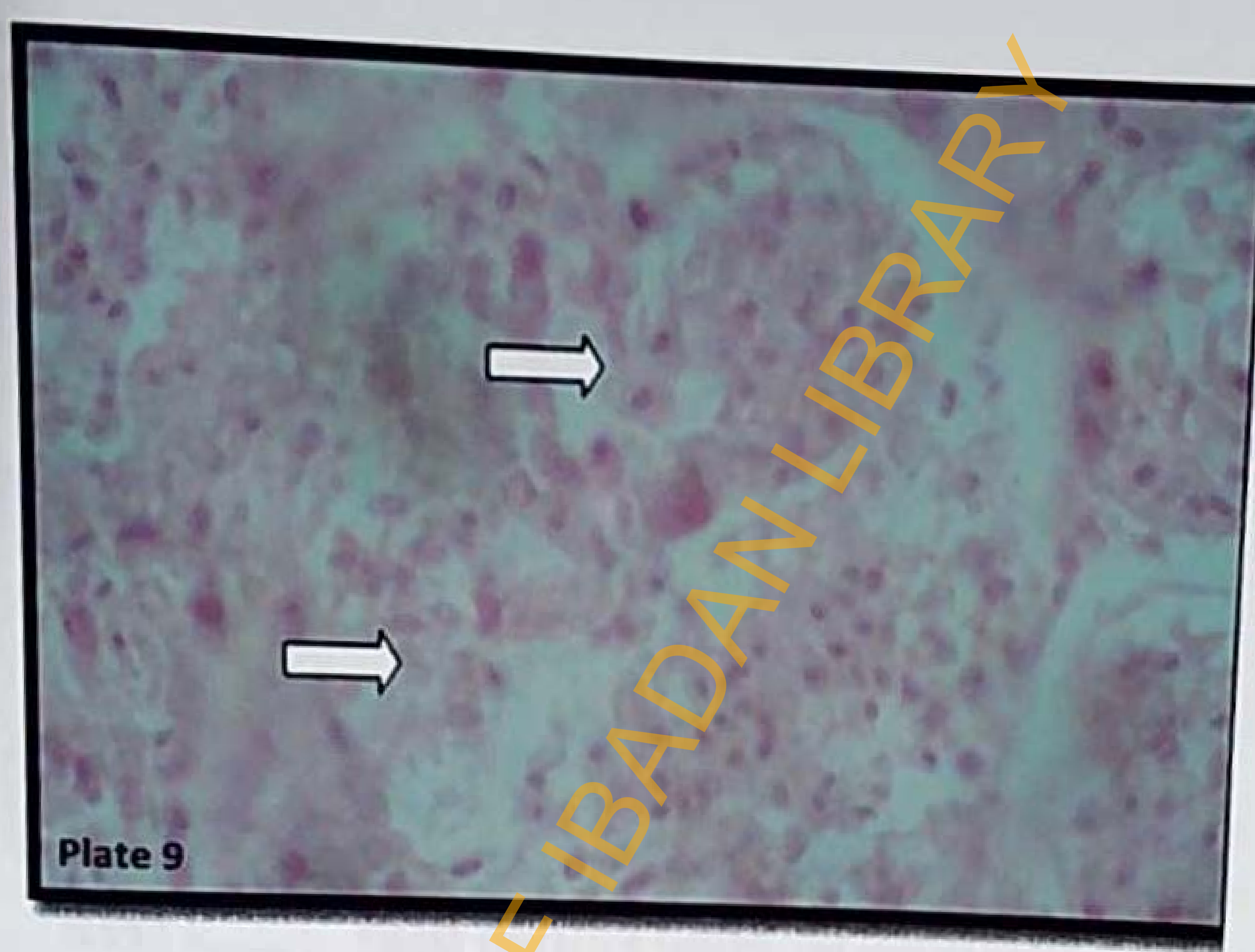

RB: ⇨ Degeneration and necrosis of germinal epithelium

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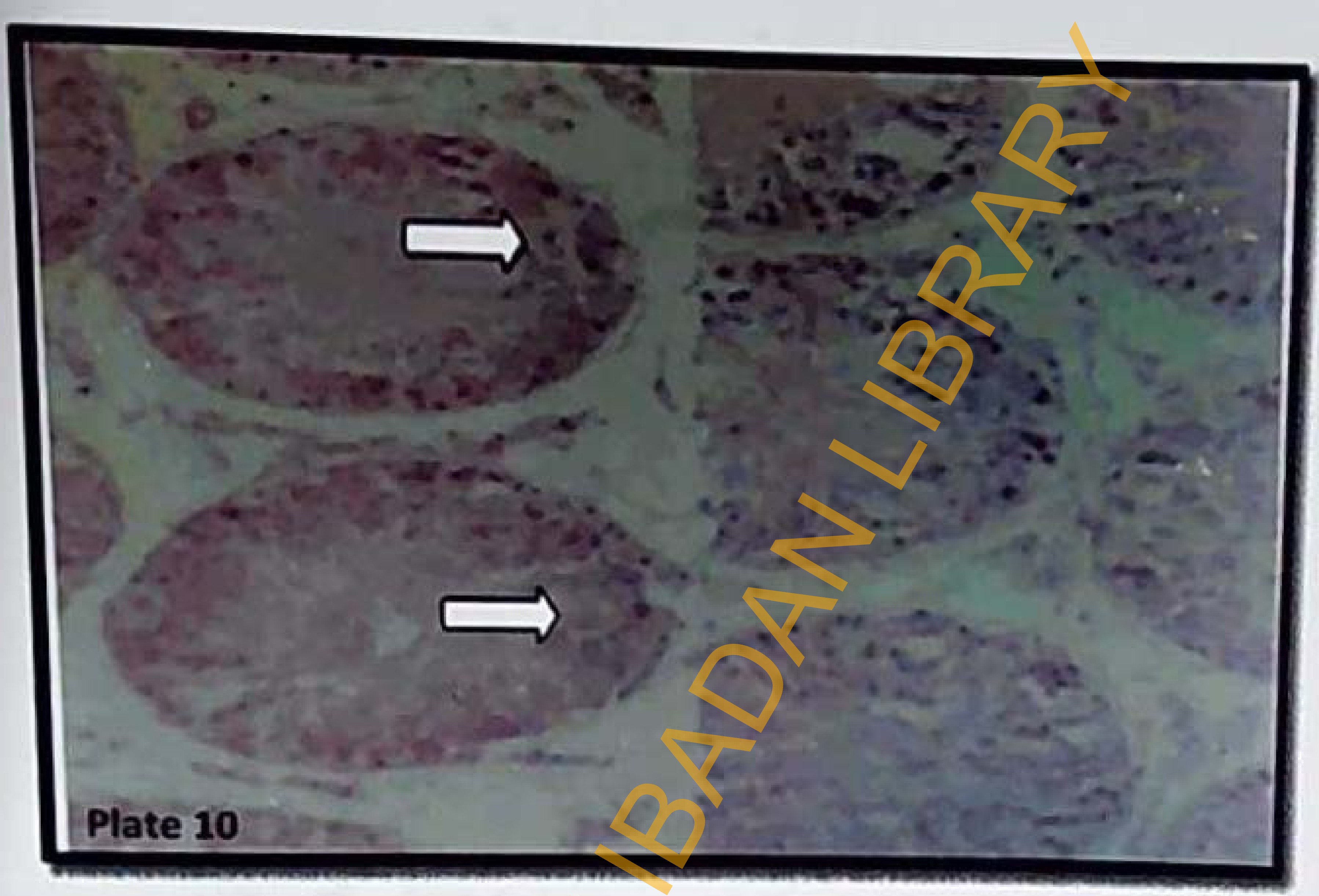

RW: ⇨ Mild diffuse degeneration and necrosis of germinal epithelium

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HB: ⇨ Mild degeneration of seminiferous epithelial cells, very few cells are present in the tubules

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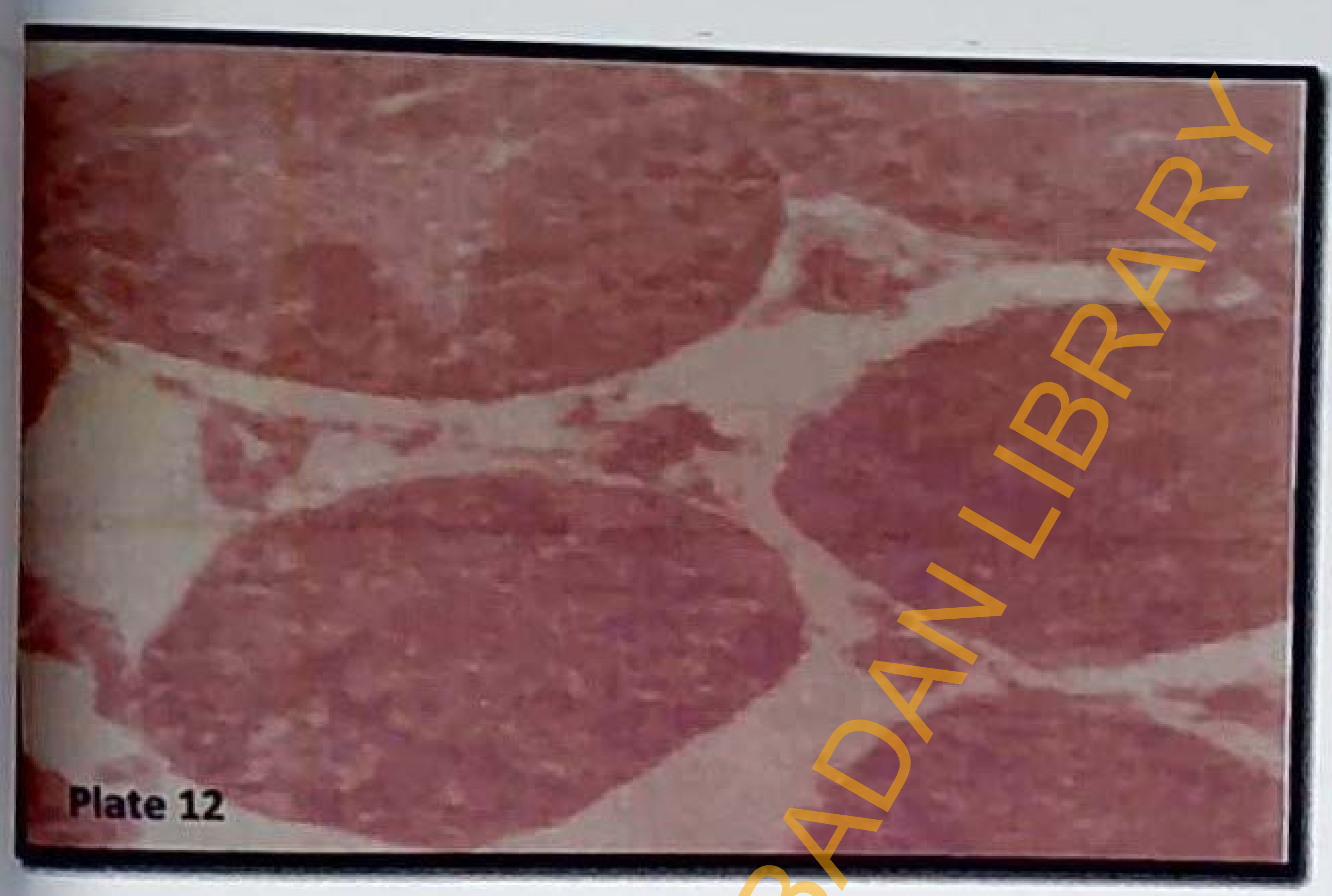

Control: No significant lesions were found

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Conclusion

The results of the haematological, serum biochemical and histopathological examinations reveal that the antinutritional/ toxic factors present in the lablab beans could have deleterious effects on the liver, kidney and testis when consumed in the raw state and efforts should therefore be made to adequately process and reduce these antinutrients/toxic factors before consumption by humans and animals.

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EXPERIMENT FIVE

4.5 Anti-oxidant and Free radical scavenging activities of the anti-nutritional factors in *Lablab purpureus* seeds.

Introduction

The greatest paradox of aerobic respiration is that oxygen, which is essential for energy production, may also be detrimental because it leads to the production of reactive oxygen species (ROS) (Saleh and Agarwal, 2002).

There is a growing body of evidence suggesting that free radicals play an important role in the development of tissue damage and pathological events in living organisms (Aruoma, 1998; Lefer and Granger, 2000; Smith *et al*., 2000; Bhatia *et al*., 2003; Olinski *et al*., 2003; Peuchant *et al*., 2004). Knowledge regarding the chemical nature and mechanisms of action of antioxidants and their important role in prevention and treatment is rapidly evolving (Neelgheen *et al*., 2006). The degree of oxidative stress in a cell depends on the concentration of the free radicals which is determined by the balance between their rate of production and their rate of clearance by various antioxidant compounds and enzymes. When free radical generation exceeds the rate of clearance, oxidative stress sets in and this in turn brings about apoptotic cell death. Oxidative stress is a condition in which the elevated levels of ROS damage cells, tissues or organs (Moller *et al*., 1996; Sharma and Agarwal, 1996; Saleh *et al*., 2003). The generation of excess free radicals have been positively correlated with increased rates of apoptotic cell death in cells (Dumont *et al*., 1999; Slater *et al*., 1995; Afolabi *et al*., 2009). ROS are free radicals that play a significant role in many of the sperm physiological processes such as capacitation, hyperactivation and sperm-oocyte fusion (Aitken *et al*., 2004; Allamaneni *et al*., 2004; deLamarinde *et al*., 1998). However, they also trigger many pathological processes in the male reproductive system and these processes have been implicated in cancers of the bladder and prostate, as well as in male infertility (Bankson *et al*., 1993; Hietanen *et al*., 1994; Agarwal and Saleh, 2002).

The toxicity of any compound in the final analysis is the sum total of its interactions with cell constituents to produce chemical alterations and the cell response to these aberrations. Whenever such disfunctions happen in a tissues, it is traceable to

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biochemical derangements at the subcellular level (DeBruin, 1976). Therefore, recourse could be made to the appropriate organ function tests to elucidate the subtle effects of these toxins even when the animal is still alive. The biochemical abnormality revealed by such biochemical tests are often obvious long before the genesis of morphological damage and precedes development of chronic degenerative disease (Kaneko, 1980). Since the liver and the kidney are often the major targets of poisonous substances, organ function tests are directed at these two organs.

Procedure

Twenty male adult Wistar albino rats (about 16 weeks) with an average weight ranging between175g to 250g were obtained from the Animal House of the Faculty of Veterinary Medicine, University of Ibadan, Ibadan. They were randomly assigned into four (4) groups of five (5) animals per group. The rats were housed in plastic cages and were housed in a conducive environment. The animals had access to clean water supply and commercial rat pellets from (Ladokun Animals Feeds Ltd) *ad libitum* during the three weeks of acclimatization. All the rats were also subjected to a natural photoperiod of about 12 hours light and 12 hours dark daily. All the experiments including animal handling and sacrifice were conducted strictly in conformation with standard guidelines of the "Institutional Ethics Committee".

The rats in the first group represented the control, and they were fed a standard diet, while the rats in the second, third and fourth groups were fed diets containing different percentages of the three varieties of Lablab beans (*Lablab purpureus*), i.e. Rongai Brown, Rongai White and Highworth Black. All the feed were calculated to contain 20% crude protein and 3 kilocalories (Iso-proteinous and iso-caloric diet). The feeding was done for 14 days.

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5.5 Anti-oxidant Studies on the *Lablab purpureus* seeds

Table 15: Serum Biochemical Parameters

Sample	ALP U/L	AST U/L	ALT U/L	Urea mg/dl	Creatinine mg/dl	Initial B.W. (g)	Final B.W. (g)
Control	426.88 ± 32.53	276.32± 5.18	184.06± 8.70	80.11 ± 4.65	1.64 ± 0.01	196.89 ± 16.02	207.81 ± 31.29 (5.55%)
Rongai Brown	*775.56 ± 52.04 (81.70%)	* 342.90 ± 6.35 (24.10%)	* 50.68 ± 2.00 (72.50%)	80.68 ± 1.00 (0.71%)	1.66± 0.03 (1.22%)	206.25 ± 32.04	*175.00± 20.04 (15.17%)
Rongai White	*674.67 ±60.87 (58.05%)	* 328.42 ± 12.33 (18.90%)	* 71.11 ± 12.20 (61.40%)	85.75 ± 0.11 (7.04%)	*1.83 ± 0.06 (11.59%)	206.25 ± 11.57	*164.06 ±12.39 (20.46%)
Highworth Black	* 690.92 ±45.19 (61.85%)	*319.47± 5.54 (15.62%)	190.09± 5.61 (3.30%)	96.07 ± 3.42 (19.92%)	* 1.91± 0.03 (16.46%)	206.25 ± 22.16	*157.75± 20.05 (23.52%)

*Significant at P<0.05

ALP: Alkaline hosphatase

AST: Aspartate Aminotransferase

ALT: Alanine Aminotransferase

BW: Body weight

All the rats fed with the three varieties of lablab beans diets gave significant increases in the level of ALP and AST. The rats fed with the Rongai brown diet recorded the highest increases in the level of ALP (775.56± 52.04U/L) and AST (342.90± 6.35U/L). The rats fed with the Rongai brown and Rongai white diets recorded significant decreases in the level of ALT (50.68± 2.00U/L) and (71.11± 12.20U/L) respectively. The rats on the Highworth black diet recorded a non-significant increase in the level of ALT (190.09± 5.61U/L). All the rats fed with the lablab beans gave increases in the level of urea and creatine. The rats fed with the Rongai white and Highworth black only gave significant increases in the level of creatinine (1.83± 0.06mg/dl) and (1.91± 0.03mg/dl) respectively.

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Table 16: Protein Concentration (mg/dl)

Organ	Control	Rongai Brown	Rongai white	Highworth Black
Liver	3.41 ± 0.02	2.71± 0.39 (20.53%)	* 2.93 ± 0.06 (14.08%)	* 2.81 ± 0.17 (17.60%)
Kidney	2.38 ± 0.04	* 1.45 ± 0.09 (39.08)	* 1.75 ± 0.04 (26.47%)	* 1.58 ± 0.22 (33.61%)
Testes	1.51 ± 0.08	1.35 ± 0.02 (10.60%)	* 1.34 ± 0.03 (11.26%)	1.33 ± 0.08 (11.92%)

*Significant at $P<0.05$

All the rats fed with the three varieties of lablab beans diets recorded decreases in the concentration of protein in the liver, kidney and testes. The decreases were not significant for the liver and testes of rats fed Rongai brown (2.71± 0.39mg/dl) and (1.35 ± 0.02mg/dl) respectively. The testes of rats fed the Highworth black recorded protein level of (1.33± 0.08mg/dl).

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Table 17: Lipid Peroxidation Assay (MDA formed/g tissue)

Organ	Control	Rongai Brown	Rongai white	Highworth Black
Liver	0.04 ± 0.01	* 0.10 ± 0.01 (150%)	* 0.13 ± 0.01 (225%)	* 0.14 ± 0.01 (250%)
Kidney	0.08 ± 0.02	* 0.16 ± 0.01 (100%)	* 0.11 ± 0.01 (37.50%)	* 0.14 ± 0.01 (75.00%)
Testes	0.09 ± 0.01	* 0.13 ± 0.01 (44.44%)	* 0.16 ± 0.01 (77.80%)	* 0.16 ± 0.03 (77.80%)

*Significant at $P<0.05$

All the rats fed with the three varieties of lablab beans diets recorded significant increases in lipid peroxidation (LPO) of the liver, kidney and testes. The rats fed with the Highworth black had the highest level of LPO in the liver (0.14 ± 0.01MDA formed/g tissue), the rats fed with Rongai brown had the highest level of LPO in the kidney (0.16 ± 0.01 MDAformed/g tissue), while the rats fed with Rongai white and Highworth black had the highest level of LPO in testes (0.16 ± 0.01MDAformed/g tissue) and (0.16 ± 0.03 MDAformed/g tissue) respectively.

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Table 18: Catalase Activity (µmoles of H_2O_2 consumed/min/mg protein)

Organ	Control	Rongai Brown	Rongai white	Highworth Black
Liver	198.72 ± 6.44	* 239.53 ± 4.97 (20.54%)	* 250.94 ± 3.07 (26.28%)	*242.50±7.65 (22.03%)
Kidney	294.28 ± 9.06	*536. 77±14.77 (82.40%)	*397.15± 12.80 (34.96%)	*474.99± 31.0 (61.41%)
Testes	455.81 ± 26.21	*564.18± 4.19 (23.78%)	*561.04± 14.64 (23.09%)	*563.40± 18.33 (23.60%)

*Significant at $P<0.05$

All the rats fed the with three varieties of lablab beans diets recorded significant increases in catalase activities of the liver, kidney and testes. The rats fed with Rongai white recorded the highest increase in the level of catalase in the liver (250.94±3.07µmoles of H_2O_2 consumed/min/mg protein), the rats fed with the Rongai brown gave the highest level of catalase in the kidney (536. 77±14.77µmoles of H_2O_2 consumed/min/mg protein) and testes (564.18± 4.19µmoles of H_2O_2 consumed/min/mg protein).

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Table 19: Superoxide Dismutase (SOD) Activity (µmoles/mg protein)

Organ	Control	Rongai Brown	Rongai white	Highworth Black
Liver	34.55 ± 7.72	45.74 ± 7.10 (32.40%)	* 56.20 ± 1.64 (62.70%)	* 63.11 ± 11.70 (82.70%)
Kidney	20.51 ± 1.46	* 28.60 ± 1.30 (39.44%)	* 13.96 ± 2.60 (31.93%)	16.17 ± 1.13 (21.16%)
Testes	10.48 ± 0.17	12.68 ± 3.16 (20.99%)	14.33 ± 2.29 (36.74%)	*25.01 ± 2.52 (138.64%)

*Significant at P<0.05

All the rats fed with the three varieties of lablab beans diets produced increases in the level of SOD activity in the liver. The increase was not significant in the liver of rats fed with Rongai brown (45.74 ± 7.10µmoles/mg protein) diet . There was a significant increase in the SOD activity in the kidney of rats fed with Rongai brown (28.60±1.30µmoles/mgprotein) diet. There was a signifant increase in the SOD activity of testes of rats fed with the Highworth black (25.01± 2.52µmoles/mg protein) diet.

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Table 20: Reduced Glutathione (GSH) (µmol/g tissues)

Organ	Control	Rongai Brown	Rongai white	Highworth Black
Liver	305.91 ± 4.01	* 285.86 ± 3.76 (6.55%)	* 282.57 ± 4.11 (8.0%)	*287.30 ± 4.50 (6.08%)
Kidney	281.45 ± 0.60	* 291.45 ± 1.10 (3.55%)	*291.73 ± 0.96 (3.65%)	*292.51 ± 7.52 (3.93%)
Testes	271.02± 0.19	272.76 ± 1.70 (0.64%)	* 276.16 ± 0.69 (1.90%)	*274.83 ± 1.01 (1.41%)

*Significant at $P<0.05$

All the rats fed with the three varieties of lablab beans diets gave significant decreases in the level of reduced glutathione of liver. The kidney of all the rats gave significant increases in the level of reduced glutathione. There were also increases in the level of reduced glutathione of the testes but the increase was significant only in the testes of rats fed with Rongai white (276.16 ± 0.69µmol/g tissues) and Highworth black (274.83±1.01µmol/g tissues).

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Table 21: Glutathione Peroxidase (GPx) (µmol/mg protein)

Organ	Control	Rongai Brown	Rongai white	Highworth Black
Liver	65.50 ± 0.04	73.01 ± 1.14 (11.47%)	*79.95 ± 0.68 (22.06%)	* 76.15 ± 0.21 (16.26%)
Kidney	97.32 ± 2.43	*211.14± 10.22 (117%)	*132.62± 3.02 (36.30%)	*165.59± 11.85 (70.15%)
Testes	150.67 ± 7.40	*168.00 ± 0.51 (11.50%)	* 172.50 ± 3.34 (14.50%)	*180.81± 0.95 (20.00%)

*Significant at $P<0.05$

All the rats fed with the three varieties of lablab beans diets gave increases in the level of glutathione peroxidase of the liver. The increases were only significant in the liver of rats fed with Rongai white (79.95 ± 0.68µmol/mg protein) and Highworth black (76.15 ± 0.21µmol/mg protein) diets. There were significant increases in the level of glutathione peroxidase of the kidney and testes of all the rats fed the three varieties of the lablab beans.

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Table 22: Glutathione-S-Transferase (GST) (µmol CDNB-GSH complex formed/min/mg protein)

Organ	Control	Rongai Brown	Rongai white	Highworth Black
Liver	0.04 ± 0.01	0.14 ± 0.04 (250%)	0.16 ± 0.07 (300%)	0.09 ± 0.02 (125%)
Kidney	0.34 ± 0.04	0.56 ± 0.10 (64.71%)	0.49 ± 0.01 (44.12%)	0.46 ± 0.11 (35.30%)
Testes	0.20 ± 0.07	* 0.60 ± 0.07 (200%)	* 0.41 ± 0.01 (105%)	0.34 ± 0.04 (70%)

*Significant at $P<0.05$

There were increases in the level of GST of the liver, kidney and testes of all the rats fed with the three varieties of lablab bean diets. However, the increases were only significant in the testes of rats fed with Rongai brown (0.60±0.07µmolCDNB-GSHcomplex formed/min/mg protein) and Rongai white (0.41±0.01CDNB-GSHcomplex formed/min/mg protein) diets.

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Table 23: Hydrogen Peroxide Generation (H_2O_2) (μmol/min/mg protein)

Organ	Control	Rongai Brown	Rongai white	Highworth Black
Liver	87.69 ± 0.55	96.93 ± 10.55 (10.54%)	*107.48 ± 2.01 (22.60%)	*103.59 ± 6.37 (18.13%)
Kidney	17.60 ± 0.42	25.61 ± 1.67 (45.51%)	18.11 ± 0.21 (2.90%)	18.53 ± 4.42 (5.28%)
Testes	75.10 ± 5.20	150.09 ± 9.44 (99.90%)	107.41 ± 6.20 (43.02%)	86.86 ± 3.11 (15.70%)

*Significant at $P<0.05$

There were increases in the level of hydrogen peroxide generation of the liver, kidney and testes of all the rats fed with the three varieties of lablab beans diets. However, the increases were significant only in the liver of rats fed with Rongai white (107.48± 2.01μmol/min/mg protein) and Highworth black (103.59±6.37dietsμmol/min/mg protein).

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Table 24: Sperm Quality in the Male Rats

Parameters	Control	Rongai Brown	Rongai white	Highworth Black
%Motility	94 ± 2.20	*62 ± 8.4 (34.04%)	*76 ± 5.5 (19.15%)	*74 ± 5.5 (20%)
%Viability	98 ± 0.00	*93.75 ± 2.5 (4.34%)	*91.25 ± 2.5 (6.89%)	* 93.75 ± 2.5 (4.34%)
TSN	36.00 ± 3.61	30.67 ± 4.04 (14.81%)	*28.00 ± 3.0 (22.22%)	*26.67 ± 6.43 (25.92%)
ESN	127.25 ± 6.08	*77.25 ± 5.12 (39.37%)	*93.75 ± 4.65 (26.33%)	*98.75 ± 6.29 (22.40%)
% Abnornality	12.06 ± 0.06	*13.67 ± 0.21 (13.35%)	*13.27 ± 0.27 (10.03%)	*13.71 ± 0.14 (13.68%)
DSP X 10^6	6.90 ± 1.56	* 7.79 ± 1.92 (12.90%)	* 6.52 ± 1.07 (5.51%)	* 5.90 ± 0.61 (14.49%)
Tesies Weight (g)	2.56 ± 0.19	* 2.21 ± 0.31 (13.67%)	* 2.18 ± 0.24 (14.84%)	2.32 ± 0.35 (9.38%)

* Significance at $p<0.05$

(%) Percentage Difference/Change

Testicular Sperm Number (TSN x10^6/cells/g tissue)

Epididymal Sperm Number (ESN x10^6/cells/g tissue)

Daily Sperm Production (DSP)

All the rats fed with the three varieties of lablab beans recorded significant decreases in %motility, %viability, TSN and ESN. The rats fed with Rongai brown gave the lowest level of %motility (62±8.4%). The rats fed with Rongai white recorded the lowest level of %viability (91.25±2.5%) while the rats fed with Highworth black gave the lowest level of TSN (26.67 ± 6.43 x 10^6/cells/g tissue). The rats fed with Rongai brown recorded the lowest level of ESN (77.25 ± 5.12x10^6/cells/g tissue). All the rats fed with the three varieties of lablab beans recorded significant increases in %abnormality and daily sperm Production. The rats fed with the Highworth black gave the highest %abnormality (13.71 ± 0.14%), while the rats fed with Rongai brown gave the highest daily sperm Production (7.79 ± 1.92 X 10^6). All the rats fed with the lablab bean diet gave significant

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decreases in testes weight. The rats fed with the Rongai white recorded the lowest value for testes weight (2.18 ± 0.24g).

Histopathology

LIVER:

Rongai Brown: Central venous and portal congestion with mild mononuclear cell Infilteration (Plate 13)

Rongai White: Mild periportal cellular infilteration (Plate 14)

Highworth Black: Multiple foci of cellular infilteration and mild hepatic necrosis (Plate 15)

Control: No significant lesions were found (Plate 16).

KIDNEY:

Rongai Brown: No visible lesions were found (Plate 17)

Rongai White: No visible lesions were found (Plate 18)

Highworth Black: No visible lesions were found (Plate 19)

Control: No visible lesions were found (Plate 20)

TESTES

Rongai Brown: No visible lesions were found (Plate 21)

Rongai White: No visible lesions were found (Plate 22)

Highworth Black: Greatly reduced seminiferous tubular diameters (Plate 23)

Control: No visible lesions were found (Plate 24)

RB= Rongai Brown; RW= Rongai White; HB= Highworth Black

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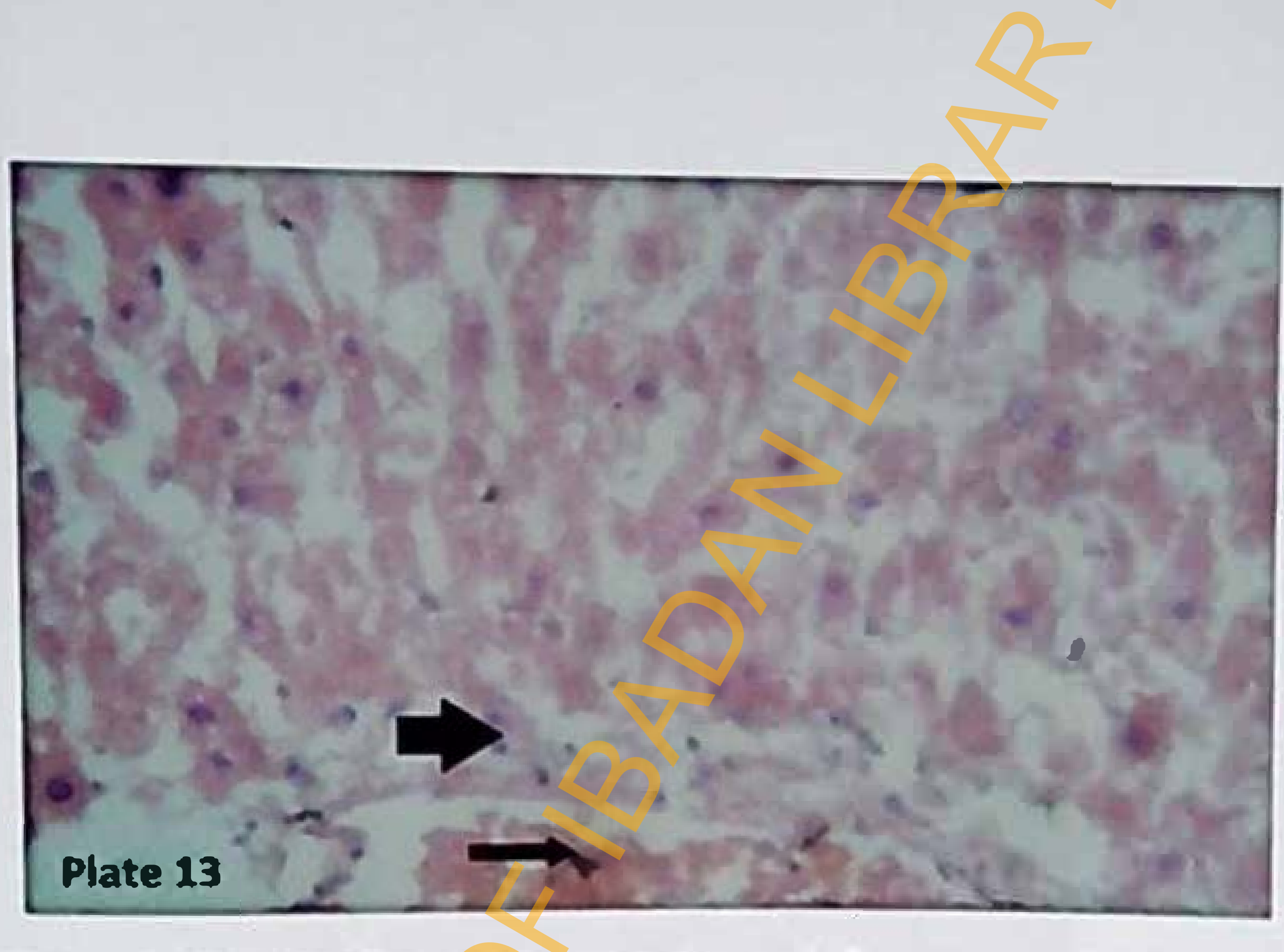

RB: → Central venous and portal congestion
➡ mild mononuclear cell infiltration.

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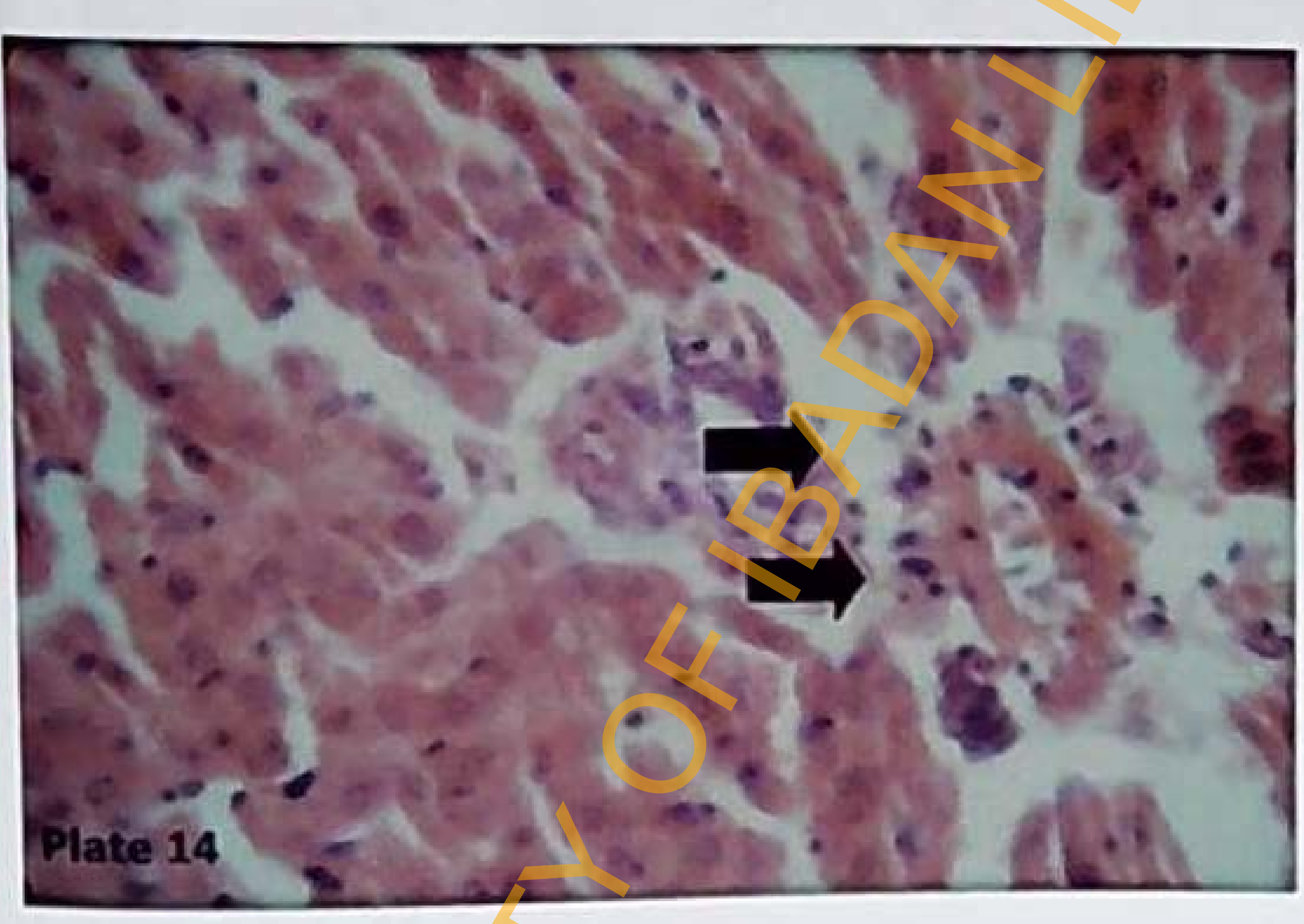

RW: ➡ Mild periportal cellular Infiltration

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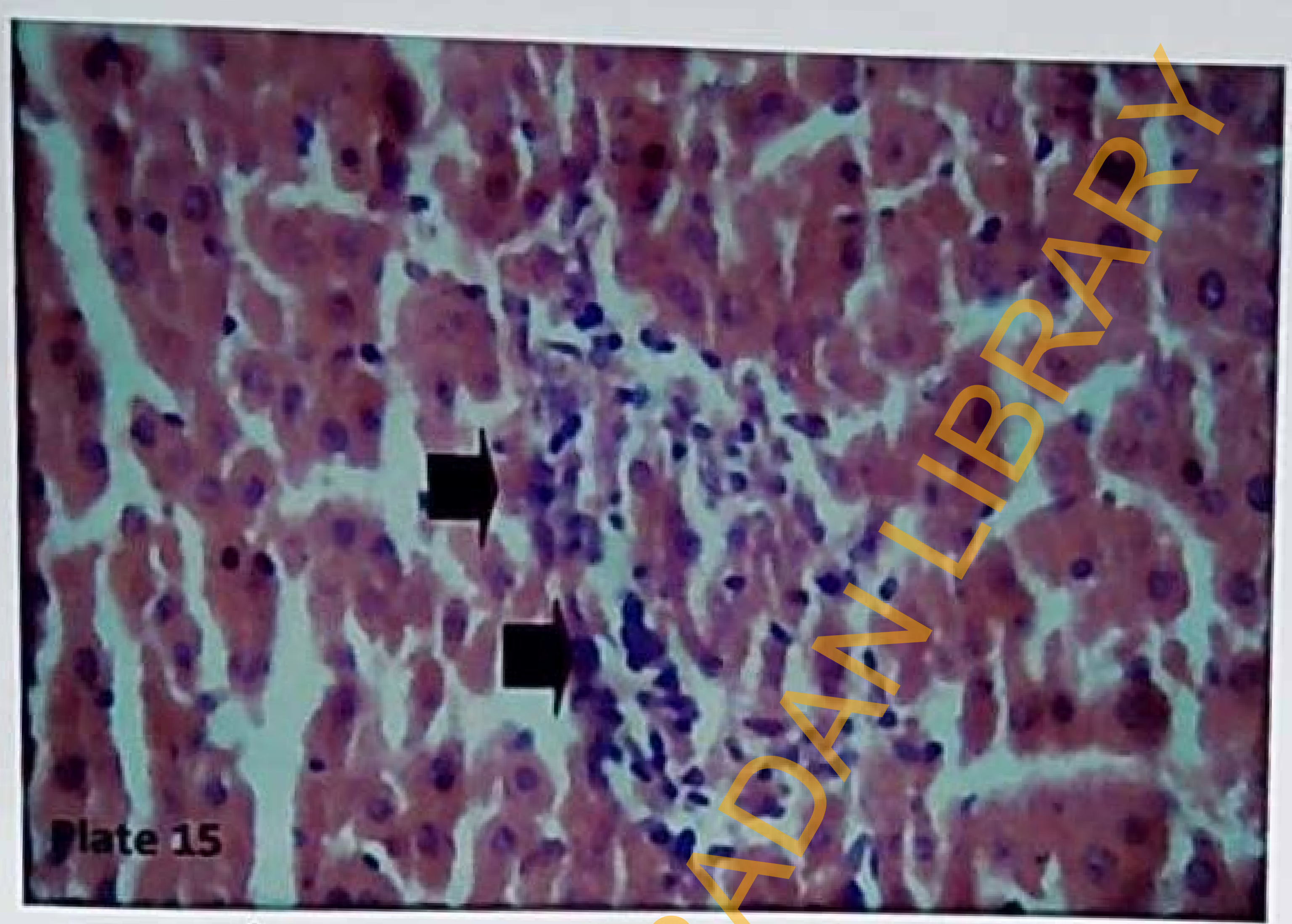

HB: ➡ Multiple foci of cellular infiltration and mild hepatic necrosis

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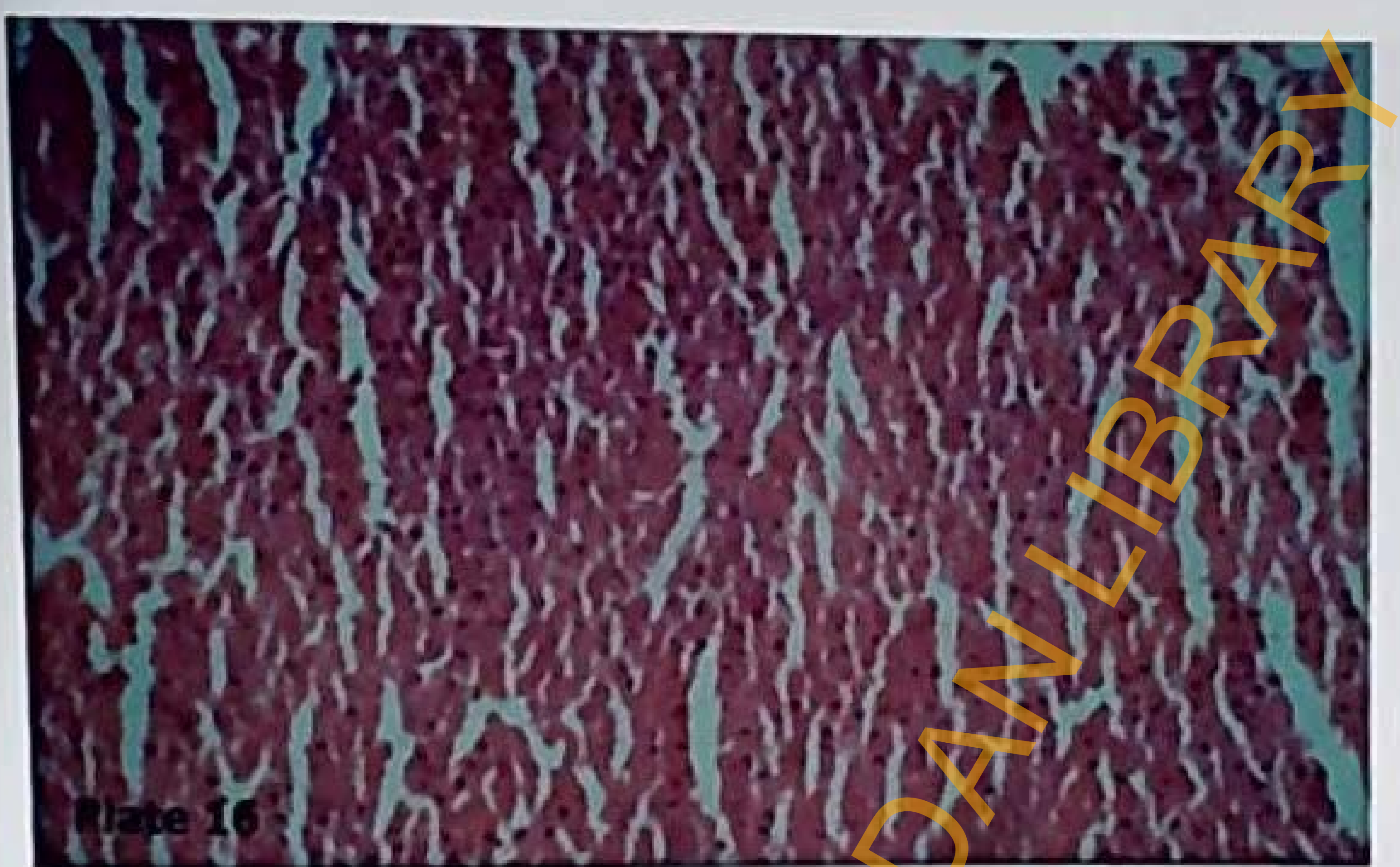

Control: No significant lesions were found

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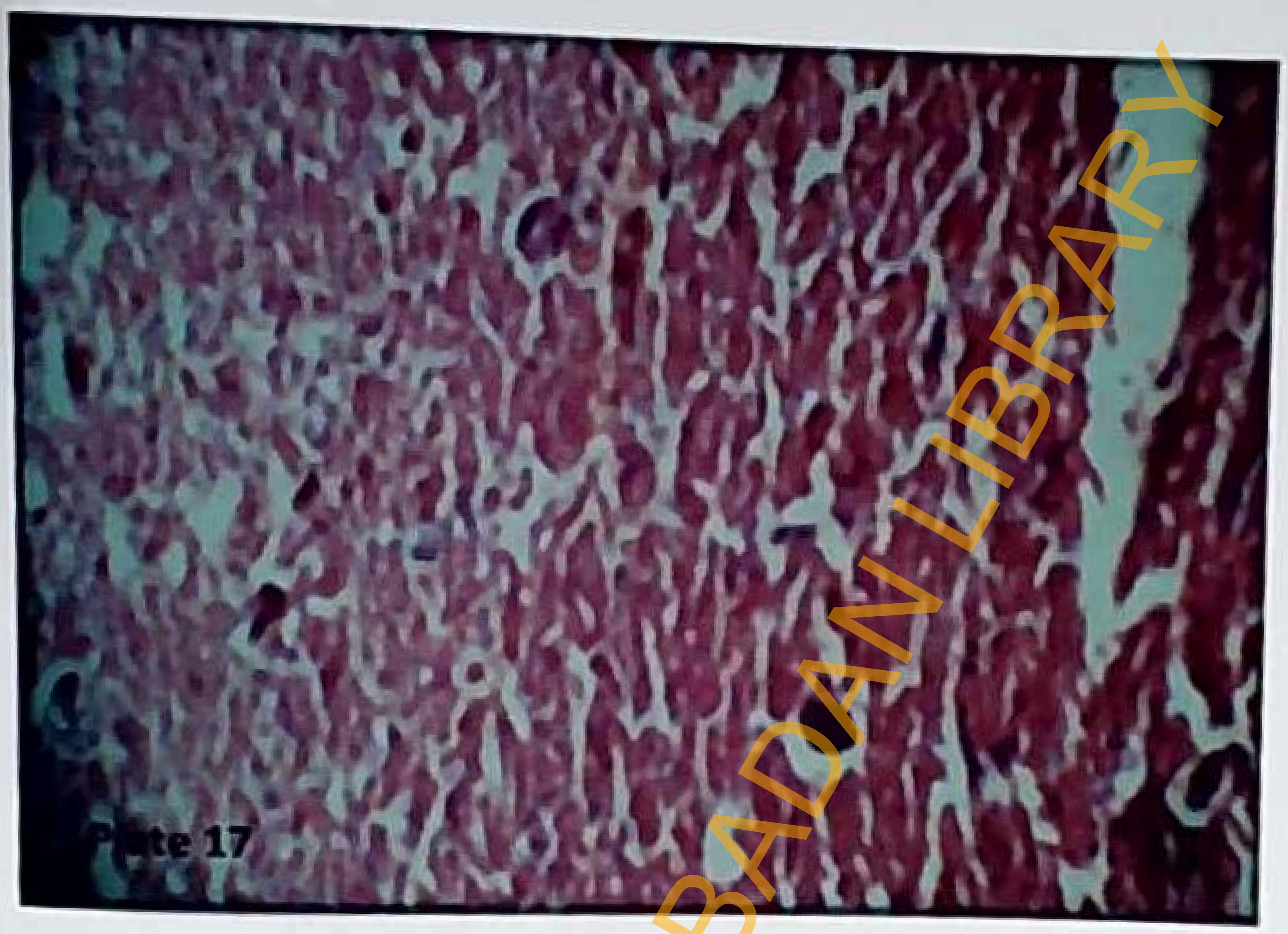

RB: No visible lesions were found

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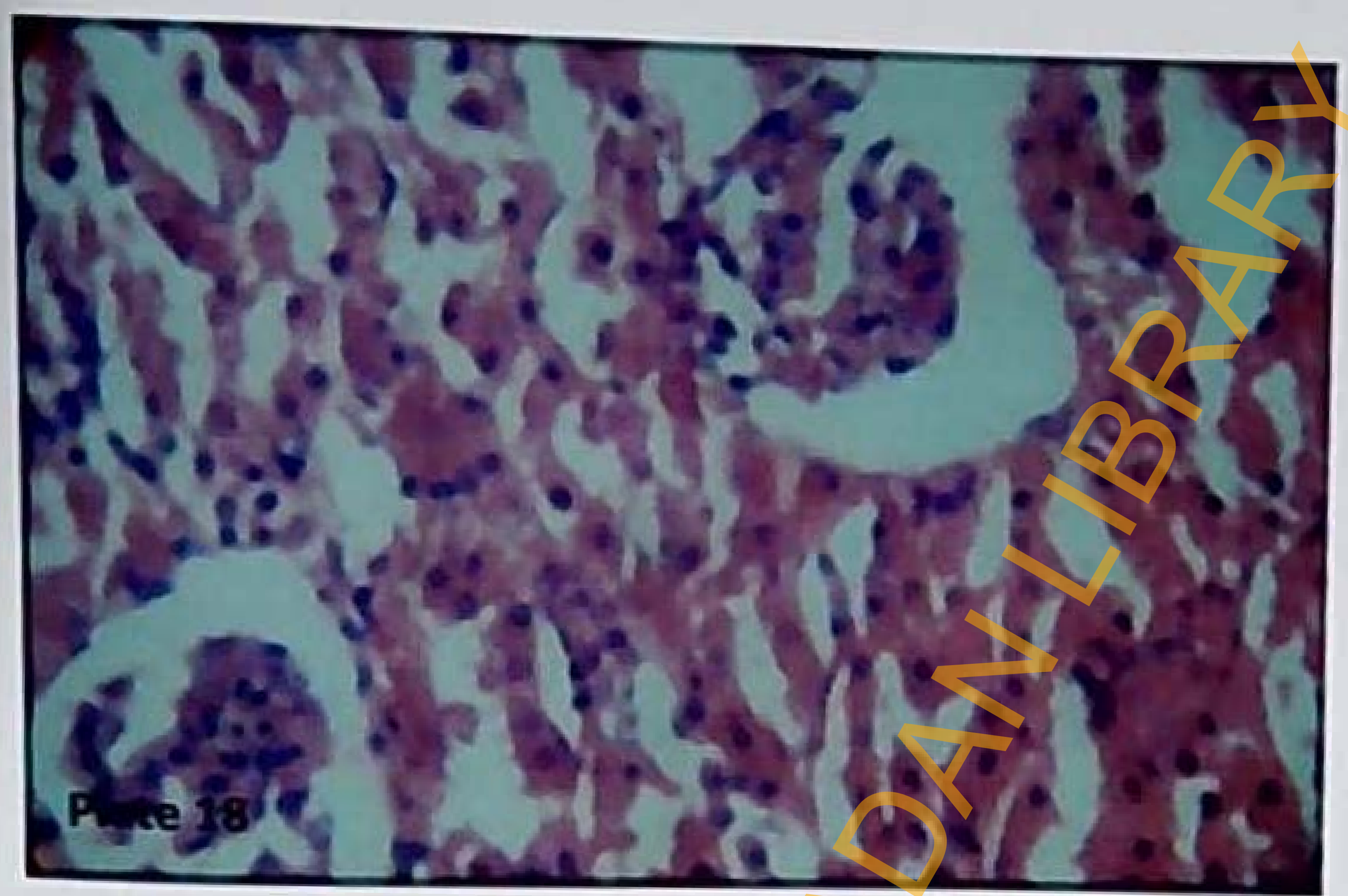

RW: No visible lesions were found

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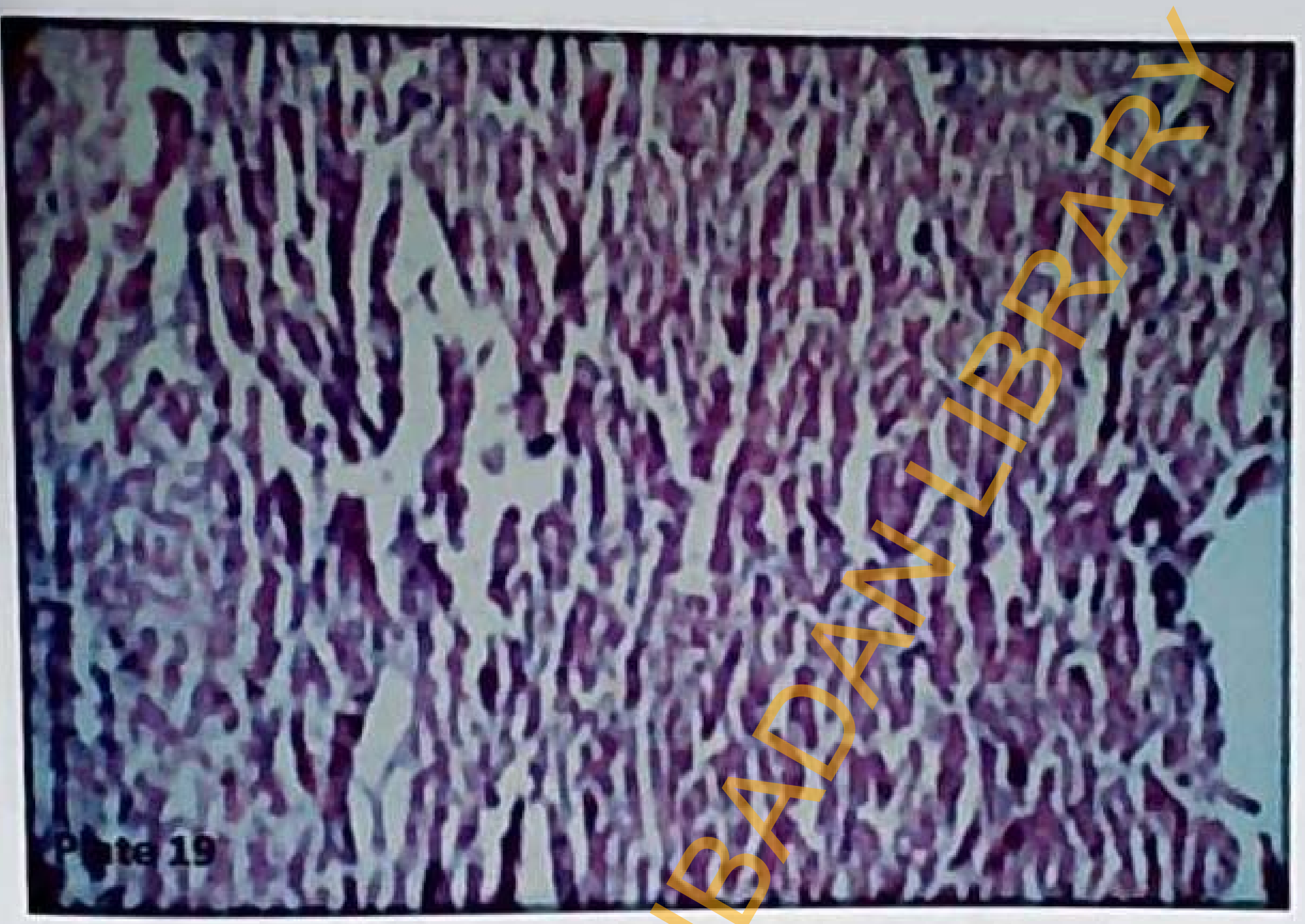

HB: No visible lesions were found

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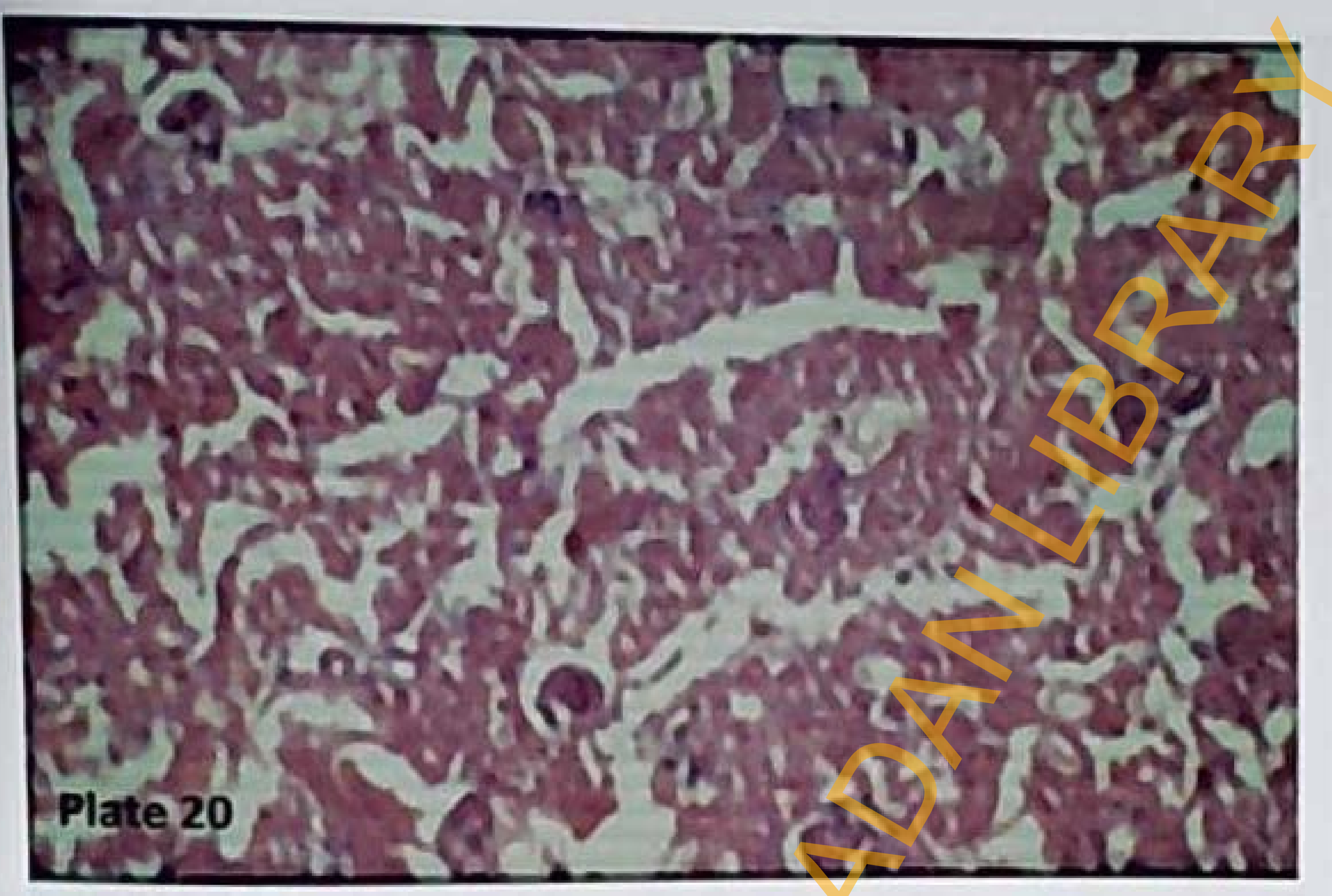

Control : No visible lesions were found

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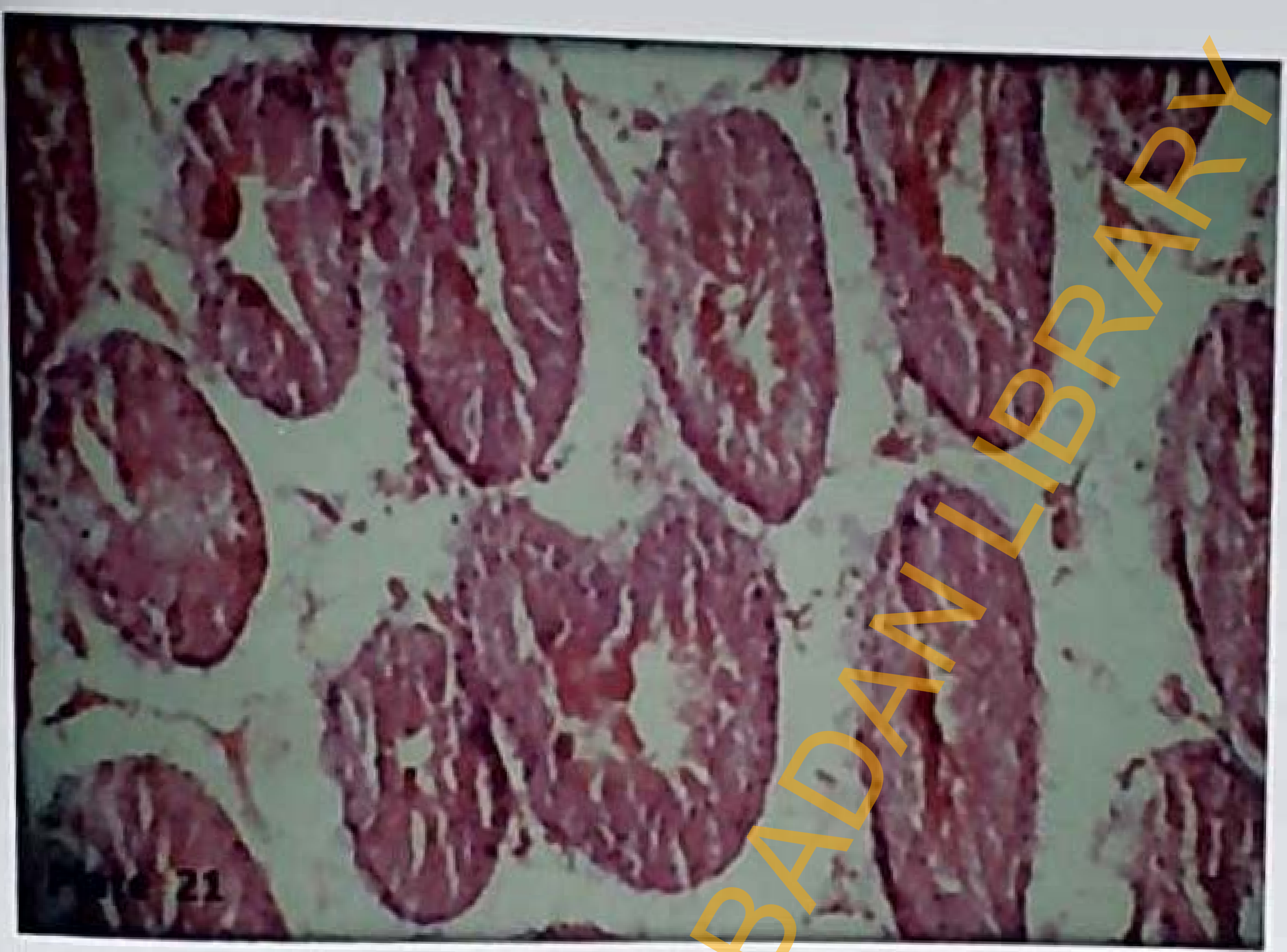

RB: No visible lesions were found

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RW: No visible lesions were found

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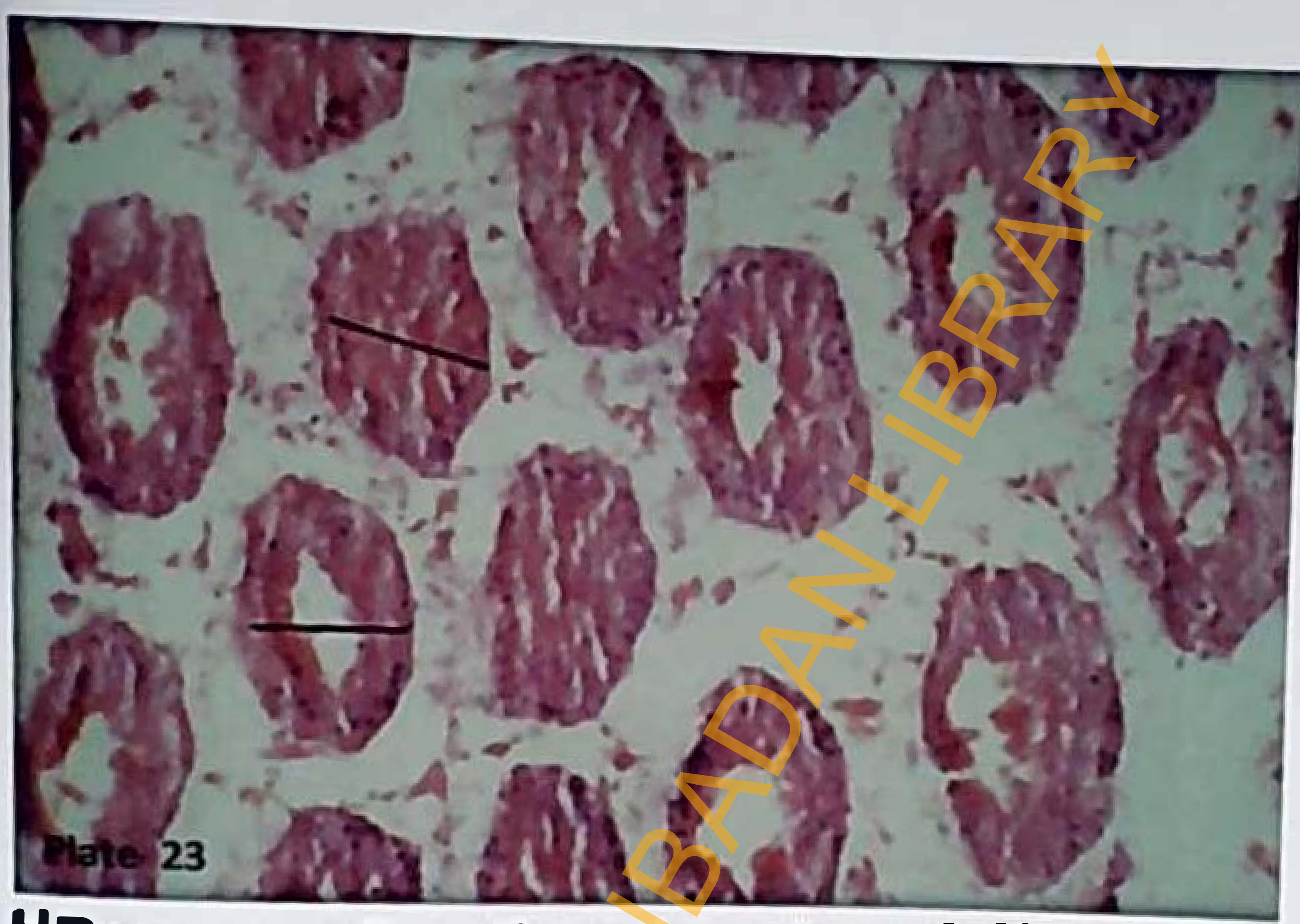

HB: ——— Greatly reduced seminiferous tubular diameters

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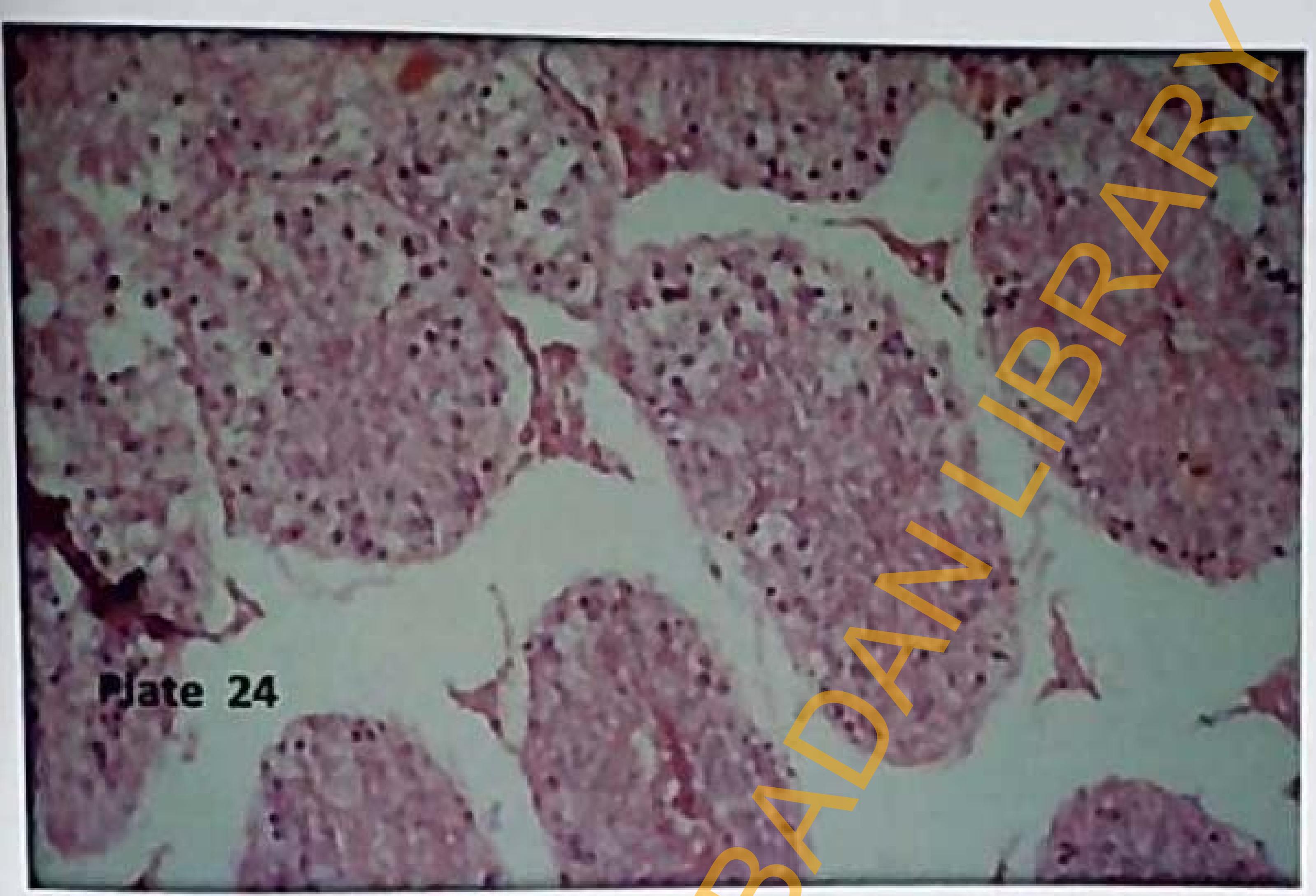

Control: No visible lesions were found

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Conclusion

The results of the antioxidant and free radical scavenging activities showed that feeding raw lablab beans to male rats induced alterations in the hepatic, nephrotic and testicular antioxidant systems, impairs testicular function, spermatogenesis and epididymal sperm physiology and morphology and thus the adverse effect of antinutritional factors (ANF) on the liver, kidney, testes and epididymis have been demonstrated.

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EXPERIMENT SIX

4.6 Molecular Weight determination of the *Lablab purpureus* seed proteins

Introduction

Since the development of sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) by Weber and Osborn, (1969), this procedure has largely replaced conventional gel electrophoresis for separating proteins (Rampton *et al.*, 1977).

The usefulness of seed protein variability for discriminating among cultivars and wild accessions as well as for studying the genetic relationships among lines have been widely reported for legume and cereal crops (Wrigley *et al.*, 1982; Brown *et al.*, 1982 and Romero-Andreas and Bliss, 1985). The usefulness of seed-protein profiles in taxonomic and evolutionary studies as well as in discriminating wild and cultivated accessions of legume species have been well established (Przybylska, 1986; Gepts *et al.*, 1986 and Lioi, 1987). Protein electrophoretic profiles have also been used to predict successful interspecific crosses (Sullivan and Freytag, 1986).

Procedure:

Electrophoresis is the migration of a charged particle under the influence of an electric field. Many important biological molecules such as amino acids, proteins, peptides, nucleotides and nucleic acids, possess ionisable group and, therefore, at any pH, exist in solution as electrically charged species as cations (+) or anions (-) (Wilson and Walker, 2004). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures qualitatively. It is useful for monitoring protein purification and, because the method is based on the separation of proteins according to size, it can also be used to determine the relative molecular weight of proteins.

The dehulled lablab beans to be run on SDS-PAGE were first boiled for five minutes in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduces the disulphide bridges present that are holding together the protein tertiary structures and the SDS binds strongly to, and denatures, the protein. The sample buffer also contains a ionisable tracking dye, that allows the electrophoretic run to be

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monitored. After loading all the samples, a current was passed through the gel. When the main separating gel (12% 0.375M tris) has been poured between the glass plates and allowed to set, the stacking gel (4% 0.125M tris) was poured on top of the separating gel and it is into this gel that the wells are formed and the proteins loaded.

The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. The stacking gel has a very large pore size, which allows the proteins to move freely and concentrate, or stack, under the effect of the electric field. When the current is switched on, the proteins separate as they pass through the separating gel, due to the molecular sieving properties of the gel. When the dye reaches the bottom of the gel, the current is turned off, and the gel is removed from between the glass plates and shaken in coomassie brilliant blue and then washed in destain solution. The destain solution removes unbound background dye from the gel, leaving stained proteins visible as blue bands on a clear background.

The molecular weights of the proteins were then determined by comparing its mobility with those of a number of standard proteins of known molecular weights that are run on the same gel. By plotting a graph of distance moved against log of molecular weight for each of the standard proteins, a calibration curve was constructed. The distance moved by the protein of unknown molecular weight was then measured, and then its log molecular weight and hence molecular weight was determined from the calibration curve.

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2 = Rongai Brown

3 = Rongai White

4 = High range marker

5 = Low range marker

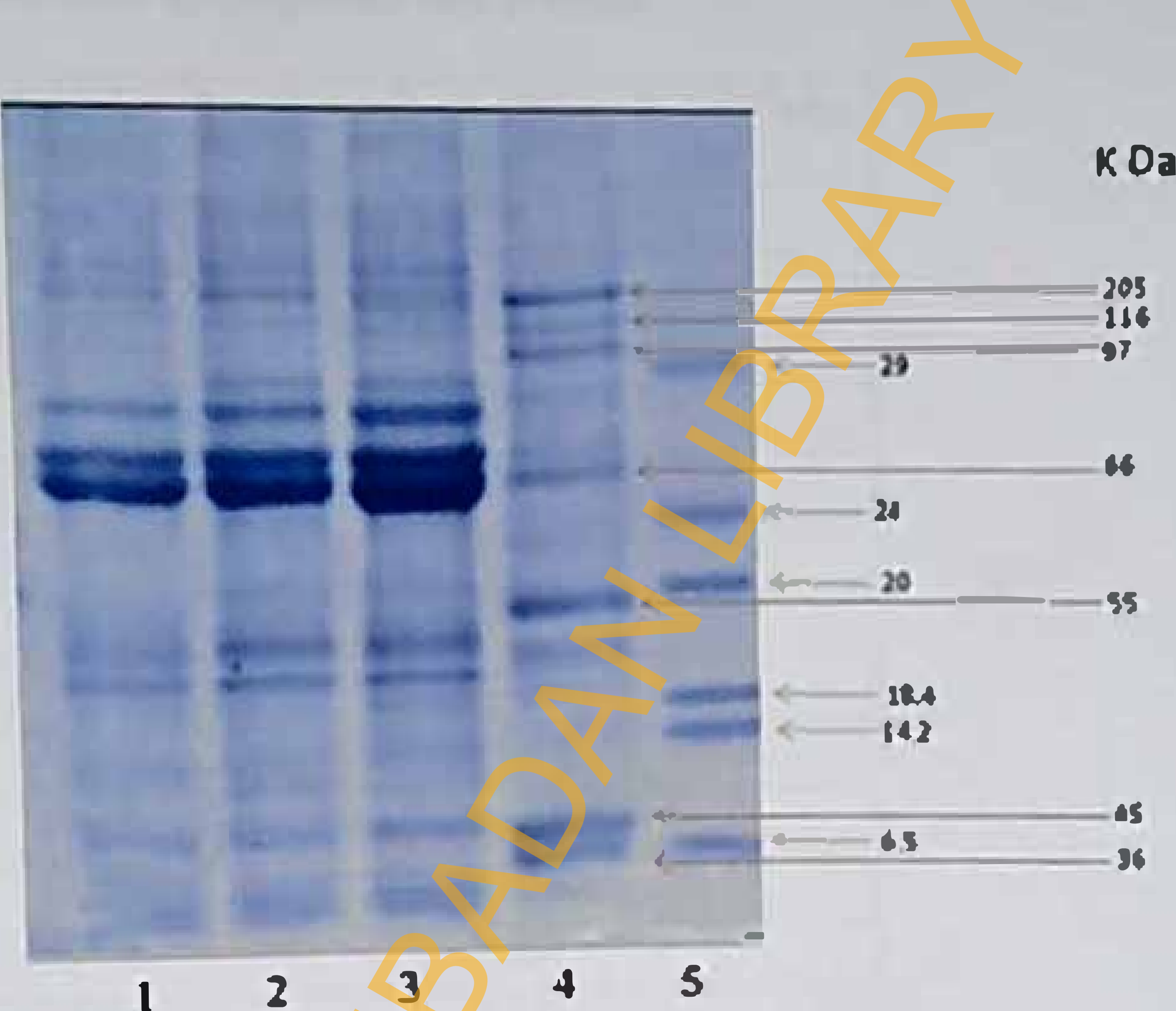

Figure 5: Electrophoregram of *L. purpureus* seeds proteins

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Table 25 Molecular weights of *L. purpureus* seed proteins

LANE 1 (Black) Daltons	LANE 2 (Brown) Daltons	LANE 3 (White) Daltons
43,652	50,118	50,118
39,811	45,709	45,709
38,019	43,652	43,652
35,481	39,811	39,811
29,512	35,481	33,884
28,840	33,884	29,512
17,378	29,512	28,840
15,849	28,840	20,417
15,136	25,119	16,596
8,709	19,498	15,849
7,943	17,378	13,182
7,244	15,136	9,120
	12,023	8,317
	10,715	8,709
	9,120	7,943
	7,943	
	7,244	

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Table 26 Comparison of molecular weights of *L. purpureus* seeds proteins

MOLECULAR WEIGHT (Daltons)	LANE 1 Black	LANE 2 Brown	LANE 3 White
50,118 •	-	+	+
45,709 •	-	+	+
43,652	+	+	+
39,811	+	+	+
38,019 ••	+	-	-
35,481	+	+	-
33,884 •	-	+	+
29,512	+	+	+
28,840	+	+	+
25,119 •••	-	+	-
20,417 ••••	-	-	+
19,498 •••	-	+	-
17,378	+	+	-
16.596	-	-	+
15,849	+	-	+
15,136	+	+	-
13,182 ••••	-	-	+
12,023 •••	-	+	-
10,715 •••	-	+	-
9,120 •	-	+	+
8,709	+	-	+
8,317 ••••	-	-	+
7,943	+	+	+
7,244	+	+	-
Summary Bands/Proteins	12	17	15

Key:

+ = present

- = absent

• (+RB & RW)

•• (+HB)

••• (+RB)

•••• (+RW)

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The results of the molecular weights of the *L. purpureus* seed proteins shown in Tables 25 and 26 above reveals that:

The proteins with the molecular weights of 43,652, 39,811, 29,512, 28,840 and 7,943 daltons were present in all the three varieties of the *L. purpureus* seeds.

The protein with the molecular weight of 38,019 daltons is present only in the Highworth black variety.

The proteins with the molecular weights of 25,119, 19,498, 12,023 and 10,715 daltons were present only in the Rongai brown variety.

The proteins with the molecular weights of 20,417, 16,596, 13,182 and 8,317 daltons were present only in the Rongai white variety.

The proteins with the molecular weights of 50,118, 45,709, 33,884 and 9,120 daltons were present in Rongai brown and Rongai white varieties only.

The proteins with the moleculor weights of 35,481, 17,378, 15,136 and 7,244 daltons were present only in Higbworth black and Rongai brown varieties.

The proteins with the molecular weights of 15,849 and 8,709 daltons were found only in Highworth black and Rongai white varieties.

Conclusion

Biochemical characterization of the lalab bean proteins revealed 17 bands for Rongai brown, 15 bands for Rongai white and 12 bands for Highworth black. The molecular weight of the proteins ranged from 7,244 to 50,118 daltons.

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CHAPTER FIVE

DISCUSSION

5.1 Proximate Analysis and Mineral Elements

Legumes are mainly noted for their high protein content relative to other plants. Results obtained show that the crude protein content of the 3 varieties of *L. purpureus* seeds were quite high, ranging between 22.75 and 24.15%. The Rongai brown variety had the highest crude protein value of 2415%, followed by Rongai white with 23.10% and Highworth black having the least value of 2275%. These values for the 3 varieties compare favourably with those values reported by Oyenuga, (1968) for cowpea seeds, pigeon pea, water melon (unshelled bambara variety) and water melon, (unshelled serese variety) which are 24.67%, 23.77%, 24.36% and 23.55% respectively. Adrain and Helias-Frangner (1964) obtained crude protein contents of 23% for bambara groundnut (*Voandzeia sibterranea*), 24.30% for the hyacinth bean and 17.45% for the cowpea (*Vigna unguiculata*), all from Senegal. The results obtained for the crude protein also agree with the findings of Amirshani and Tavakoli (1970) who obtained an average protein content of 24.28% and the range of 22.45 to 27.25% based on 47 varieties of cowpea.

Ologhobo (1980) also reported crude protein content ranging between 24.33 and 27.09% in the cowpeas and 20.59 and 25.88% in the lima beans. Apata and Ologhobo (1994), Ene-Obong and Carnovale (1992) reported that the crude protein content of legumes ranges generally from 20-40%. Of all legumes species, the soyabeans are the richest in terms of protein content (43%), while other legumes have crude protein content range of 20-25% (Apata and Ologhobo, 1994; Ene-Obong and Carnovale, 1992; Swaminathan and Jain, 1973).

The different genetic origins of the 3 varieties of lablab beans must be partly responsible for the varietal differences observed. Swaminathan and Jain (1973) reported that differences in nutrient contents of legumes are dependent on the variety and location. In an experiment conducted in several locations using a number of varieties of grain legumes, extensive differences were observed between varieties and locations.

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Smirnova-Ikonnikova (1962) observed significant variations in the accumulation of proteins in the seeds of different varieties of cowpea and soyabeans and suggested that the nature of these changes must depend on specific peculiarities and growing condition of the legumes. The importance of the lablab beans as a protein supplement for animal feed and its potential value for human food is therefore well borne-out/highlighted by these experiments. Reports by Oshodi and Ekperigin, (1989) and Aletor and Aladetimi, (1989) showed that pigeon pea (*Cajanus cajan*) contain a moderate level of crude protein ranging from 21.2-22.5% in the three varieties studied. The crude fat content of the three varieties of *L. purpureus* seeds were moderately high when compared with the cowpea, lima bean and pigeon pea varieties. Rongai brown has the highest crude fat content having 9.74%, followed by highworth black 9.63 and Rongai white 9.56.

The cowpea varieties have a range of between 0.52 and 2.88% and the lima bean varieties ranged between 0.51 and 2.99% as reported by (Ologhobo. 1980). However soyabean varieties recorded very high crude fat content of between 21.59 and 26.62% according to Ologhobo and Fetuga, (1984c). Osagie *et al.*, (1986) reported crude fat content of between 0.9 to 2.1% for lima beans. Dela-Vega and Sotelo (1986) reported crude fat content ranging between 0.9 to 2.8% for lima bean. Temple *et al.* (1991) reported crude fat content of 19.45% for soyabeans. Pigeonpea has crude fat content ranging between 1.7 to 2.3% for the 3 varieties studied as reported by Ene-Obong and Carnovale (1992). Although there are some doubts as to the nutritional significance of crude fat in the legume diet, it has been established that it is a normal source of linoleic and linolenic acids, which are two of the essential fatty acids required by the animal body. The minor constituents of the legume crude fat have been reported to include carotenoids (Provitamin A), vitamins D, E, and K. In addition to its role in curing night blindness, vitamin A has been implicated in protein synthesis (Baurenfeind, 1972).

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Legumes, except the oil legumes, are low in crude fat content, ranging from 1 to 5%. However, oil seeds have a range of crude fat contents from about 18% in soyabean to as high as 43% in groundnut (Apata and Ologhobo, 1994; Ene-Obong and Carnovale 1992; Swaminathan and Jain, 1973). Of interest are the crude fibre contents of the lablab beans varieties which ranged from 12.69% to 13.12%. Rongai white has the highest 13.12%, followed by Highworth black 12.98% and Rongai brown 12.69.These values are very high when compared to that of other food legumes. Ologhobo (1980) recorded crude fiber range of between 4.85 to 6.65% for lima beans and between 2.43 to 3.98% for cowpea varieties. These values were also more than those reported for kidney bean Pondo 6 (4.0%), lima bean TPL88 [5.4%] Yara-1 (5.4%), TPL 249 [4.5%] pigeonpea Ex-Ibadan (4.8%), TUC 5537-1 (4.4%) by Apata and Ologhobo, (1994) and also for soyabeans (4.28%) reported by Temple *et al*, (1991) and those reported for good grade cashewnut meal (0.69 to 1.23%) and groundnut cake (4.53%) by Fetuga *et al*. (1973ab;1974)

These values are not considered too high when compared with values reported for other feeding stuffs, like jack bean (9.5%) by Apata and Ologhobo (1994), African locust bean (8.76 to 12.64%) by Fetuga *et al*. (1973ab). The high crude fiber contents of the lablab bean varieties do not disrecommend them as undesirable for non-ruminants on basis of reported claims. Eastwood, (1974), reported that there is a relationship between the absence of fiber in the diet and the incidence of a wide range of diseases in man notably colon diverticular, diabetes mellitus, obeisity and coronary heart disease. The presence of fibre in the diet increases the bulk of the faeces, which has a laxative effect on the gut.

The studies of McConnel *et al* (1974), suggest that dietary legume fibre derives its physiological action from its roles in water retention within the gut. The high ash contents in the lablab bean varieties could reflect as high mineral elements. The ash content ranged from 3.97 to 4.28%. These results were within the normal range reported for cowpea 3.6-4.4%, pigeon pea 3.6-4.1% and African yam bean (*Sphenostylis stenocarpa*) by Ene-Obong and Carnovale (1992).

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Ologhobo (1980) recorded mean ash content of 4.24% for cowpea varieties and 5.06% for lima bean varieties studied and this reflected on the high mineral contents of these legumes. The Nitrogen free extract (carbohydrate component) of the lablab beans ranges between 39.1 to 40.39%. These values compare favourably with that reported for cowpea seeds estimated to be between 45 and 60% by Onigbinde and Akinyele, (1983).

Carbohydrates are known to contribute the greatest quota of energy required by man and animals for the maintenance of the various metabolic processes. The availability of these carbohydrates is important for energy utilization by monogastric animals and man for which legumes form part of the staple diet. The strategic position of legumes as food sources in the future must be accorded its rightful place. Currently, legumes have become the hidden component in most foods and also very important industrial material. There is therefore the need to improve on its production. The results of mineral elements content showed slight variations among the lablab seeds varieties. The relative proportions of these minerals in a specific kind of legume may, however, be of more important consideration since some minerals may be highly concentrated, leading to the detrimental deficiencies of others that may be essential. The most abundant element in all the three varieties of lablab seeds is potassium, ranging from 1.52% in Highworth black to 1.61% in Rongai brown. When these legumes are consumed in adequate amounts, they could provide sufficient potassium to meet the needs of an individual and thereby prevent the danger of muscular paralysis, mental disorientation and cardiac irregularities often associated with a fall in the level of potassium in the plasma (Apata, 1990).

Meiners *et al.* (1976b) have reported that legumes contain low amounts of sodium. This is confirmed in the present study. The sodium content ranged from 0.16% in Rongai white to 0.18% in Rongai brown. The low sodium content in these legumes suggest that they can be important food in low sodium diets recommended for hypertensive patients since high sodium diet is implicated in cardiovascular and renal disorders. However, in an otherwise normal person with a sufficiently high rate of legume intake, other dietary sources of sodium such as common table salt, might be

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necessary as a supplement (Apata, 1990). This is needed in the tropical countries where much sodium may be lost through sweat as sodium chloride.

The phosphorus content ranged from 0.42% in Rongai white to 0.48% in Rongai brown. Bressani and Elias, (1974) obtained value of 0.30% for phosphorus in edible leguminous seeds, and they were classified as poor sources of this essential mineral. The value reported for phosphorus in the present study were above 0.30% and this indicates them to be good sources of this mineral.

The calcium contents of the raw lablab seeds ranged between 0.66% in Highblack to 0.70% in Rongai brown. The review by Patwardhan, (1962) revealed legumes to be comparatively poor sources of dietary calcium. The magnesium content ranges from 0.31% in Rongai white to 0.33% in Rongai brown. Manganese content ranged from 0.06% in Rongai brown to 0.07% for both Highworth black and Rongai white. The concentration of iron ranged from 155 parts per million (ppm) in Rongai white to 168 ppm in Rongai brown. Literatures reported that legumes are good sources of iron (Gophalan *et al.*, 1978; Tropical legumes, 1979).

The presence of mineral elements in animal feed is vital for the animal's metabolic processes. Grazing livestock from tropical countries often do not receive mineral supplementation except for common salt and must depend almost exclusively upon forage for their mineral requirements (McDowell *et al.*, 1984). Mineral deficiencies and imbalances in soils and forages account partly for low animal production and reproductive problems (Akinsoyinu and Onwuka, 1988). Minerals in the diet are required for metabolic reactions, transmission of nerve impulses, rigid bone formation and regulation of water and salt balance among others (Adejumo and Awosanya, 2005). There is very limited information on mineral elements of some legumes consumed in Nigeria especially the lesser known legumes (Agunbiade, 1992). This study has shown that *L. purpureus* seeds contain appreciable quantities of the mineral elements.

Studies have shown that *Lablab purpureus* combines a great number of qualities that can be used successfully under various conditions. Its first advantage is its adaptability, not only is it drought tolerant, it is able to grow in a diverse range of

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environmental conditions worldwide (Cameron, 1988). Staying green during the dry season, it has been known to provide up to six tones of dry matter per hectare. Of all the three varieties, Rongai brown has the highest concentration of crude protein, crude fat, ash and gross energy although it has the lowest value of crude fibre and nitrogen free extract (NFE). However, only the crude protein, crude fibre and NFE produced significant difference at $p<0.05$.

General concern has been shown in the sustainance of the normal healthy person and in nutrition in relation to disease. Attention has also been drawn to the alarming inadequacies, both in quantity and quality in the world's food supply (FAO, 2008) and in the imbalance between growth in population and food production. The interest to produce foods with good high quality protein content is steadily increasing in various countries (Bressani *et al.*, 1978). Due to the high cost of animal protein sources like meat, egg etc from cattle and poultry, attention is diverted to provision of dietary protein from vegetable sources which are much more available to the low-income groups of developing nations (Agunbiade, 1992).The world production figure of grain legumes was estimated to be 51 million tones in 1976, but this figure was considered to be underestimated by between 20 and 50%, since in the tropics and subtropics, many of the legumes are grown and consumed at rural level and do not enter official statistical reports (Kay, 1979). The concerted efforts of various research centres, notably (I.I.T.A. 1998) are geared towards increased cultivation and improvement of vegetable proteins especially the grain legumes. Almost too much attention has been paid to the popular legumes that the significance of other legume grains is almost obscure (Ezueh, 1977). Molina *et al.*, (1977) have emphasized the need for vigorous research on non-conventional legumes for use in food preparations as protein sources.

With the current levels of interest and commitment to the production, storage and utilization of legumes in Nigeria in particular, and worldwide in general, legumes are poised to be the food of the future in feeding the teeming populations and an important protein source for the poorer countries of the world. Lablab beans is one of the legumes well sited to accomplish this based on the preliminary studies on the proximate analysis.

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Food legumes provide between 17 and 40% of protein in diets (Bressani and Elias, 1980; Jood *et al.*, 1986).

This study reveals that *Lablab purpureus* has some qualities and potentials above other common legumes like the cowpea, soyabean and pigeonpea, as manifested in its high crude fibre and crude protein content. Efforts should therefore be devoted to conducting more research to extend both technical and practical knowledge about *Lablab purpureus* so that its full potential may be achieved.

5.2 Antinutritional factors

All the results reported here are consistent with the view that legumes, though rich in proteins, are sources of anti-nutritional factors, some of which are toxic and, when fed raw, limit the nutritive usefulness and protein digestibility of such legumes (Aletor and Fetuga, 1984b). The trypsin inhibitor activities in the raw lablab bean varieties are summarized in Table 20 above. The results of the trypsin inhibitor activities in the raw lablab bean reveal that they contain considerable amount of these inhibitors. Rongai brown has the highest value of trypsin inhibitor activities while Rongai white recorded the least and trypsin inhibitors are reported to be one of the major toxic components of legumes (Liener and Kakade, 1980; Akinyele, 1989).

Trypsin inhibitors adversely affect protein utilization in two ways (i). In chicks, it inhibits intestinal proteolysis by decreasing the effective level of trypsin to form an inactive trypsin-trypsin inhibitor complex (Kakade *et al*, 1970). (ii). In rats, it increases the requirement of sulphur containing amino acids, thus accentuating the deficiency of the amino acids which already exist in the plant (Kakade *et al*, 1970). The major antinutritional factors implicated in reducing the nutritional qualities and potentials of legumes are the trypsin inhibitors, haemagglutinins and hydrogen cyanide (Ologhobo, 1980). These trypsin inhibitor values compare favourably with the values obtained for soyabean ranging from 35.30 and 36.90 tiu/mg protein and lima bean ranging from 29.43 to 36.65 tiu/mg protein as reported by Ologhobo (1980) but exceed those obtained for cowpea ranging from 19.60 to 28.20 tiu/mg protein as reported by Ologhobo and Fetuga (1983). The results are also consistent with that reported for cowpea ranging from 19.0 to 46.7 tiu/mg protein by (Marconi *et al.*, 1993).

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The trypsin inhibitor activities obtained for the lablab beans are contrary to the results reported by Lambert (1972,) for 26 varieties of cowpea (*Vigna sinensis*). The authors obtained a range of 15.5 to 23.80tiu/mg protein and went on to show that environmental and genetic factors affect the trypsin inhibitor activities of legumes. The results of the Heamagglutinin content reveals that Highworth black has the highest HU/mg while Rongai white records the least. These results compare well with that recorded for some varieties of cowpea TVNU66 having 13HU/mg and TVNU226 having 27HU/mg as reported by (Marconi *et al.*, 1993).

The results from this study, however, contradict those of Ologhobo and Fetuga (1983) reporting a mean value of 49.6HU/mg protein for cowpea (*Vigna unguiculata*). However, there appears to be no basis for comparison in as much as the isolation Procedures and methods employed differ. Furthermore, the varieties of legumes used and even the environment in which these legumes were grown might have some modifying influences, as reported by Swaminathan and Jain (1973). The variability within species and between locations may be as high as 10%. For the cyanogenic glycosides, Highworth black has the highest with Rongai white recording the least. The occurrence of cyanogenic glycosides in legumes was reported by (Okolie and Ugochukwu, 1989). The cyanogenic glycosides on hydrolysis yield toxic hydrocyanic acid (HCN). (Fernando, 1987; Osuntokun, 1972). The cyanide ions inhibit several enzyme systems, depress growth through interference with certain essential amino acids and the utilization of essential nutrients. The results obtained for lablab beans is lower than that reported by Egbe and Akinyele (1990) reporting 420mg/kg cyanogenic glycoside in lima bean (*Phaseolus lunatus*). Okolie and Ugochukwu (1989) showed total cyanide contents of 381-1095mg/kg for seeds of Nigerian varieties of *Phaseolus aureus*, *Vigna unguiculata*, *Cajanus cajan* and *Canavalia gladiatus*. These are higher than that reported for *Lablab purpureus* seeds. The differences observed in the varieties of lablab beans and different species of legumes could be attributed to the genetic variations and different nutrient composition of the soils on which they were cultivated. Similarly, macro and micro mineral deficiencies in the soil have been found to result in the non-

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protein nitrogen pool being large and this could result in elevated cyanogenic glycoside levels (Butler *et al.*, 1973).

For the oxalates, Highworth black recorded the highest while Rongai white has the least. Oxalates (C_2 dicarboxylic acid anion) is produced and accumulated in many crop plants and pasture seeds. Oxalates may be present in plants as the soluble salts, potassium, sodium or ammonium oxalate as oxalic acid or as insoluble calcium oxalate. Oxalate is a concern because high oxalate diets can increase the risk of renal calcium absorption. Although data are scarce in literature with which to compare our results as regards oxalates in legumes, our results agree with total oxalates reported by Aremu, (1989) for *Lophira alata* (9.85mg/g), *Hyphaene thebaica* (9.57mg/g) and *Bixa orellana* (8.44mg/g) which are wild underutilized crop seeds in Nigeria. For the phytates, Rongai white has the highest while Rongai brown has the least. Phytic acid, a hexaphosphate derivative of inositol is an important storage form of phosphorus in plants. It is insoluble and cannot be absorbed in the human intestines. Phytates can render metals like calcium, iron, zinc and magnesium unavailable into the body by forming insoluble salts with these metals (Edman and Forbes, 1977). However, our results contradict that obtained by Ologhobo (1980), recording a range of 2.90mg/g to 3.25mg/g phytate for cowpea.

For the tannins, Rongai brown recorded the highest while Rongai white recorded the lowest. Our results compare well with that recorded for lima beans tannin ranging between 0.32 to 0.93mg/g by Ologhobo (1980) but it is higher than that recorded for cowpea ranging between 0.24 and 0.58mg/g and soyabean varieties ranging between 0.34 and 0.37mg/g by Ologhobo (1980).

Not much information appears to exist in the literature on the toxic effects of tannins in legumes. Some earlier studies have, however, revealed that the occurrence of tannins in plants are of no nutritional significance unless at very high levels often 10% or more of the dry weight. Butler (1989) reported that tannins may decrease protein quality by decreasing digestibility and palatability. Egbe and Akinyele (1990) found 0.59mg/g tannins in raw lima beans (*Phaseolus lunatus*). The results obtained for lablab beans suggest that they very insignificantly affect the nutritional potentials of these legumes.

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For the saponins, Highworth black has the highest while Rongai brown has the lowest. Saponins have been shown to possess both beneficial (Cholesterol-lowering) and deleterious properties (cytotoxic; permeabilisation of the intestine and to exhibit structure dependent biological activities (Price *et al.*, 1987; Oakenful and Sidhu, 1989). Our result is similar to that reported by Achinewhu (1983) recording 11.8mg/g saponins for rubber seed (*Hevea brasiliensis*). Our results are also similar to that reported by Osagie *et al.* (1996), that cowpea contains 11.18mg/g saponin.). Sodipo and Atinze (1985) reported beans to contain a considerable amount of saponins of about 245.0mg/kg. While there are suggestions that the consumption of saponins should be encouraged because of their hypocholesterolaemic activity, forage saponins have been reported by Cheeke *et al.* (1978) to cause toxic and anorexic effects in the rat and swine, thereby limiting the feeding value of high-saponin animal feeds such as alfalfa.

Lastly, for the alkaloids Highworth black has the highest while Rongai white has the least. Alkaloids are basic natural products occurring primarily in plants. It has been reported that probably 10 to 20% of all higher plants contain alkaloids (Wink, 1993). Literature is scarce on the levels of alkaloids in food legumes. Osagie *et al.* (1996) reported that alkaloid containing species and varieties may have been eliminated by choice from Nigeria's staple foods because of the bitter taste associated with alkaloids.The values recorded for the alkaloids are low when compared with that of other anti-nutritional factors in lablab beans. This may be an advantage to the nutritional significance of the lablab beans. Two considerations do justify continued surveillance and future research on antinutritional factors in foods and feeding stuffs. Firstly, the introduction of new legume varieties into our diets may expose humans and animals to new toxic factors with unsuspected biological effects. Secondly, improper processing of beans and pulses may expose humans to high concentrations of these toxic factors e.g. production of soyamilk and its use as an alternative to cow's milk in infant formula.

It is obvious from the results obtained for the different anti-nutritional factors in this legume seed that these antinutrients occur in the different varieties of *Lablab purpureus* seeds. Analytical Investigations and results obtained from them are important In breeding programmes. Agronomic practices could also be incorporated into breeding

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programmes, to oversee the effects of soil nutrients on the anti-nutritional factors without any adverse effects on the levels of other essential nutrients.

5.3 Phytochemical Screening

The presence of secondary metabolites of plants like saponins, tannins, alkaloids etc in *L. purpureus* seeds justifies an in-depth study on this food legume. Many triterpene saponins and their aglycones have been reported by Hostettmann and Marston (1995) and Ndukwe *et al.* (2005) to have varied uses as anti-inflammatory, antipyretic, fibrinolytic, analgesic, anti-ulcerogenic and anti-oedema agents. Condensed tannins extracted from various forages can significantly reduce the viability of the larval stages of different nematodes of sheep and deer (Molan *et al.*, 2000a, b). Triterpenoid saponins from Zygophylum species were also used in traditional medicine as an anthelmintic agent (Ibrahim and Nwude, 1983). Plants constitute an important source of active natural products which differ widely in terms of structures, biological properties and mechanisms of actions. Various phytochemical components especially polyphenols (such as flavonoids, phenolic acids, tannins etc) are known to be responsible for the free radical scavenging and anti-oxidant activities of plants (El Diwani *et al.*, 2009). Phenolic substances possess many biological effects. These effects are mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelating transition metals (Bahman *et al.*, 2007).

In recent years, the extract of many plants have been screened for their antioxidant activities. In model systems, antioxidants are able to scavenge free radicals and thereby prevent the free radicals from causing damage (El Diwani *et al.*, 2009). The presence of these secondary metabolites could be responsible for the use of the whole plant of *L. purpureus* in folk medicine as antispasmodic for fevers and stomach complaints, and the use of its leaves to treat colic and gonorrhoea and as a poulticing for snakebites (FAO, 1988). In Malaysia, mixture of *L. purpureus* leaves, rice flour and turmeric are used on eczyma, the pod juice are used to treat inflamed ears and throats (FAO, 1988). In China, it is traditionally used to supplement spleen functions, expel dampness, disperse summer heat and remove heat (Chagxiao and Peigen, 1993).

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Ndukwe *et al.* (2007) reported that the presence of saponins in *Vitellaria paradoxa* (Gaertn F.) could be responsible for the traditional use of its kernel fat extract (shea butter) as a muscle relaxant and in the treatment of sprains, wounds and colds as generally practiced in Nigeria. Phytochemical progress has been aided enormously by the development of rapid and accurate methods of screening plants for particular chemicals. These procedures have shown that many substances originally thought to be rather rare in occurrence are almost universally distributed in the plant kingdom (Banso and Adeyemo, 2007). Plants contain biochemically and physiologically active principles that over the years have been exploited in traditional medicine for the treatment of various ailments (Adebajo *et al.*, 1983). The drugs contained in medicinal plants are known as active principles. Cowman (1999) and Banso and Olutimayin (2001) reported that plants contain a wide variety of active principles.

5.4 Nutritional / Protein Quality Studies

In this study, rats fed on the raw lablab beans elicited considerable weight loss, and poor Protein Efficiency Ratio (PER) values. These clearly indicate the high potency of the toxic substances in the raw state of these legumes. The poor protein utilization indices observed in the lablab beans indicate the rats maintained on these legumes could not efficiently utilise the dietary protein.

Our results on growth parameters are corroborative of previous studies (Rubio *et al*, 1990; Apata, 1990) that the ingestion of raw navy bean, lima bean and faba bean significantly depressed growth, feed consumption, and nitrogen retention. Pusztai *et al* (1979) reported that the inclusion of raw beans in rat diets decreased protein utilization. A derangement in digestion and or absorbtion of some dietary nutrients in rats fed raw lima bean is reported by Alctor and Fetuga (1984a). From their observations, it is conceivable that the growth inhibition of rats fed raw lima bean may in part be mediated by the apparent inability of the experimental animals to utilize dietary nitrogen fully and perhaps other components in the diet as well.

The following factors have been claimed by different workers to affect the nutritional qualities of plant proteins, they are fertilizer and manorial treatments of plants. Thus heavier treatments result in more nitrogen content in the leaves and this

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could lead to increased nitrogen content of the extracted protein and increased nitrogen content of protein concentrates have been found to give higher digestibility values (Byers, 1961, Horigome and Kandatsu, 1968). Minor quantitative differences in the amino acid spectra of the leaf proteins could also affect their nutritional values (Singh, 1970a) but Pirie (1963) pointed out that this value is more limited by the digestibility values of proteins than by their amino acid composition. Raymond and Tilley, (1956), however noticed that differences in availability of some essential amino acids of the proteins, like lysine, led to differences in the nutritional qualities of the proteins from many plant species. When fed as the sole protein source to rats during bioassay, the *L. purpureus* seeds do not support growth, ostensibly because of the polyphenol levels (tannins) and other antinutrients in the seeds which may, in part, lead to poor utilization of the constituent amino acids. By implication, these seeds may not support growth if fed solely as the protein source to man.

Nigeria along with most West African countries, has a low protein intake and kwashiorkor is quite common. The development of seed protein projects in this area is both desirable and feasible. The high rainfall encourages the growth of plants. The two main reasons for protein malnutrition in Nigeria have been listed as ignorance and poverty (Bassir, 1964). This view is also shared by other reknowned Nigerian nutritionists (Oke, 1966). In an attempt to solve this problem, there is need to improve the nutritive quality of locally-available foodstuffs through better processing and enrichment (Akinrele, 1967). This study is concerned with the study of nutrient content of the three varieties of lablab bean. Locally, food legumes are obviously a choice as they are widely eaten. Although legumes are eaten by humans and animals, the high fibre content and the presence of toxic and or antinutritional factors limit the quantity that can be ingested. If the proteins are removed from the fibrous matter and concentrated, larger quantities could be safely consumed. Legume seed protein concentrate has good potential as a protein supplement because of its high yield per unit area of land, the relatively simple method of preparation and its good nutritive value. In comparison to cereal grains, food legumes contain more than twice the protein and yield

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almost as many calories per unit weight. Compared with foods of animal origin, most of these legumes contain similar amounts of protein (Akinjayeju and Enude, 2002).

Since proteins constitute one of the most important nutrient present in very high amounts in the lablob seeds, methods could be developed to prepare protein isolates and various other products for diverse end uses but the antinutritional factors have to be reduced drastically. The search for protein sources is of great importance in developing world where the population is growing at an accelerated rate. Plant proteins, such as legumes, will certainly play an important role in meeting the protein requirement of man as well as feeding his livestock (Apata, 1990). In the world's search for high quality but cheap sources of protein and energy foods, increasing attention has been focused on home-grown legume seed crops which possess potential for significant contribution of nutrients particularly proteins, that can be used to improve the diets of the vast majority of people as well as in balanced and economic rations for livestock (Apata, 1990). Information on the nutritive status of these legumes are vital for their most effective utilization and a basis for their nutritional enhancement.

The National Academy of Sciences of the United States (1984) and Achinewhu (1982) have drawn attention to several lesser-known legumes that are promising grain legumes indigenous to the tropics. Among them are the African yam bean (*Sphenostylis stenocarpa*), bambara groundnut (*Voandzeia subterranean* (L), kidney bean (*Phaseolus vulgaris*), tropical lima bean (*Phaseolus lunatus*), swordbean (*Canavalia plagiosperma*), jackbean (*Canavalia ensiformis*) and lablab bean (*Lablab purpureus*) and they are all cultivated in different localities in this country. Balogun and Fetuga (1986) classified them as underexploited leguminous crop seeds in Nigeria. These legumes have primary importance for supplementing the protein-deficient diets generally prevailing among Nigerians of low economic status but their nutritional possibilities have not yet been fully realized and exploited (Apata, 1990). They are also called minor legumes, probably because they do not enjoy a wide popularity or simply because their importance is under-estimated (Agunbiade, 1992). Mature dry legumes are very important in the diets of many population groups around the world (Meiners *et al.*, 1976a). Legumes should be exploited as food supplements because of their potentials as

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rich protein sources (Peace *et al.*, 1988). Much of the available information on the nutritive compositional data on the more commonly used local foods and feeds does not cover all these legumes and where available, needs updating. This is because of possible effects of the soil, climate, variety, processing and fertilizer-treatments on their chemical composition (FAO, 1968).

5.5 Toxicological studies

It is well known that many of the plants in our environment are toxic to some extent. It is therefore necessary to take caution in the consumption of plants (Ewar and Hall, 1978). An encyclopaedia of plants that have either being reported or suspected to cause poisoning in humans and livestock at one time or the other is important so as to reduce the occurrence of poisoning in our environment (Clarke and Clarke, 1975; Hall, 1977 and Kinghorn, 1979).

Nigeria has a poor disease-reporting system; as a result of this, the incidence and occurrence of plant poisoning is given little or no relevance. Due to the transhuman nature of livestock management in Nigeria, it is reported that about 10% of all grazing livestock are severely affected by toxic plants yearly (Nwude, 1976). The nature of such losses vary from directly causing death, emaciation or management problems in animal grazing to indirectly causing abortions or congenital deformities in the unborn generations of the affected animals (Keeler, 1973). The toxicity of plants depends on some factors. For example, *Amaranthus retroflexus* is able to acquire dangerous level of toxicity only when grown on nitrate-rich soil or when the pasture is fertilized by artificial manures containing sodium, potassium or ammonium nitrate (Clarke and Clarke, 1975) i.e. enviromental factors do affect poisoning. Also, handling affects the toxic constituents of plants. For example, *Melilotus alba* is reported to produce toxicity only when it has spoiled (Frasser and Nelson, 1959, Prier and Derse, 1962). Fasting also increases the susceptibility of animals to poisoning (Clarke and Clarke, 1975).This observation is important since livestock in Nigeria are exposed to reduced availability of pasture in the dry season when there are no rainfalls; and they have to trek long distances in search of pasture.

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The effects of many poisonous substances often manifest by the production of typical clinical symptoms and gross and histopathologic lesions in the tissues. The lesions of poisoning are reported to be rarely characteristic. Nevertheless, the findings at autopsy are reported to provide definite clues to the nature of the poison (Clarke and Clarke, 1975). The poisons, when in sufficient concentrations, kill the tissue which they come in contact with; or, if the action is milder, injure the tissues and initiate an acute inflammatory reaction. If the poison has been ingested, it is the alimentary mucous membrane which suffers the necrosis or inflammation. The most powerful ones destroy the lining of the mouth or oesophagus as they are swallowed and then carry their effect to the stomach. Others pass through the stomach with little damage and cause superficial necrosis and inflammation in the upper or lower intestine presumably because they remain longer in contact with the injured part (Smith *et al*., 1974). Furthermore, some poisons are known not to have immediate action but are absorbed to produce their action on the delicate epithelial cells of such parenchymatous organs as the liver, or kidney which they reach by the blood streams. The skin and visible mucous membranes may have a characteristic discoloration. Jaundice has been reported as a frequent sign of hepatic damage by plant poisons (Jenkins, 1963; Hutchinson, 1977; Tennant *et al*., 1981 and Dwers *et al*., 1982). A cherry red or pink colour is seen in cyanide and carbon monoxide poisoning. Methaemoglobinaemia due to nitrates, nitrites or chocolates may impart a brown colouration (Andrade *et al*., 1971). The animals showed typical toxic symptoms like crouching appearance, forehead swelling, sluggishness and general loss of fur.

All the rats that were fed the control feed exhibited progressive increases in body weights as shown in Table 14. All the rats that were fed the Rongai brown, Rongai white and Highworth black varieties showed progressive decrease in body weights. This could be due to the poor absorbtion and utilization of the feeds caused by the actions of the anti-nutritional factors. Through the portal veins, the liver is the first organ to receive substances from the gastrointestinal tract and the first to be exposed to ingested toxins. One of the functions of the liver is to detoxify and eliminate toxic substances and metabolites (Jones *et al*., 1997). The alkaloids and lectins (haemagglutinins) in the seeds

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may be the major antinutritional factor responsible for the hepatic lesions. Jones *et al.* (1997) reported that plants containing alkaloids produce hepatic lesions. According to Kakade and Evans (1965) lectin toxicity can cause zonal necrosis and fatty infilteration of the liver, oedema, marked congestion of capillary vessels and haemorrhage in various tissues.

Alkaloids, especially members of the pyrrolizidine group, are also reported to cause livestock loss due to liver lesions by grazing on plants poisoned by the alkaloids (Smith and Culvenor, 1981; Woo *et al.* (1988); Roeder, 1995; 2000 and Stegelmeier *et al.*, 1999). The difference in the hepatic lesions produced by the three varieties of lablab seeds may be idiosyncratic in nature. The toxicity of hepatotoxins varies unpredictably between animals according to Jones *et al.* (1997). The kidney lesions may be caused by oxalates and lectins in the seeds. Jones *et al.* (1997) reported that oxalates cause nephrotic lesions in the kidney. The mechanism involved in the aetiology of this toxicosis is that calcium or sodium oxalates are ingested by animals by eating plants rich in these oxalates. Radostits *et al.* (2007), also reported that oxalates may cause kidney lesions, he stated that oxalates are also known to cause nephrotic syndrome, azotaemia and kidney lesions. Ikegwuonu and Bassir (1977) also reported that lectin toxicity can produce histopathological changes of some organs, particularly in the kidney. Lectins also cause an increase in tissue invasion by normal innocuous gut bacteria due to reduction of body defences.

The lesions in the lungs may be caused by the alkaloids and cyanogenic glucosides as reported by (Smith and Culvenor, 1981; Woo *et al.*, 1988; Roeder, 1995, 2000; Stegelmeier *et al.*, 1999) that pyrrolizidine alkaloids are highly toxic to livestock and causes death due to pulmonary lesions after grazing on plants containing such alkaloids. Liener, (1989) also reported that cyanogenic glucosides cause respiratory failure and goiter. The testicular lesions may be caused by all the antinutritional factors. According to (Clause, 1997 and Noakes *et al.*, 2001), testicular degeneration and production of subfertile spermatozoa may be caused by any factor(s) which damages the testicles such as infections, heat, malnutrition or poor digestion and absorption of nutrients, toxins, age, hormonal deficiencies and obstruction to flow of sperm. The

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histological integrity of the testis is fundamental to the production of fertile spermatozoa (Oyeyemi *et al.*, 2008). The aetiology of pathological lesion to the testes have been traced to the presence of alkaloids (Burkill, 1994). Alkaloids are bioactivated to release reactive metabolites, which bind to cell molecules and cross-link DNA to cause cellular damage (Cheeke, 1988). Alkaloids also cause gastrointestinal disorders Aletor (1993a), tannins cause decreased palatability and feed consumption, binding of dietary protein and digestive enzymes to form complexes that are difficult to digest, Roeder (1995), saponins produce haemolysis and other toxicities to rats, Johnson *et al.* (1986), haemagglutinins interfere with digestion and absorption of nutrients, Aletor and Fetuga (1987b), phytates and oxalates bind essential minerals and make them unavailable (Nelson *et al.*, 1968; Oke, 1969).

The effects of poisons which are most often looked for, in plant toxicology in animals are their gross and histologic actions on the tissues. The lesions of poisoning are rarely characteristic, nevertheless, the finding at autopsy can provide definite clues to the nature of the poison (Smith *et al.*, 1974). The poisonous plants cause an array of different symptoms, since the effect of the poison is often on not more than one body system. Clinical evidence is reported to be only of limited value. There are only about nine systems of organs in the body that are capable of being affected, so the permutations and combinations of clinical signs are extremely limited. In addition, there is extraordinary variability shown by different individuals in the symptoms caused by the same poison and not every symptom is known to appear on each occasion (Zook and Gilmore, 1967). The absence of lesions may be as important as their presence as it serves to exclude various toxic agents. The skin and visible mucous membrane may have a characteristic discolouration. Jaundice is a frequent sign of hepatic damage which has been noted in various plant poisoning such as *Aspergilus flavus*, *Heliotropium europaeum* (Smith *et al.*, 1974), a cherry red or pink colour is seen in cyanogenic plant poisoning and methaemoglobinaemia due to nitrates and nitrites impart a brown colouration on the haemoglobin. In this study, the clinical signs observed in the rats fed the raw *Lablab purpureus* seeds are starry haircoat, dullness, anorexia (inappetance), weight loss, enlargement of the abdominal cavity, recombency, comatose and death.

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The various nutritional and histopathological effects of raw lablab beans on the rats system reported herein are all indications of some of the toxic properties of this bean when ingested in the raw state. Although the toxicities of most other edible legumes when consumed in their raw states have been fairly well studied, those of lablab beans have been little studied (Deka and Sarkar, 1990). Also the biochemical and pathological effects of lablab bean (*Lablab purpureus*) anti-nutritional factors have not been well documented. Andrea and Pablo (1999) also recommended that efforts must be devoted to conducting more research to extend both technical and practical knowledge about *Lablab purpureus* so that its full potential may be achieved. These two series of needed information on the lablab seeds have been provided in this investigation and this is the rationale of this aspect of investigations.

The present results with raw lablab beans agree with those of Beko *et al.*, (1972); Santidrian *et al.* (1981) who showed that growing rats and chicks fed diets containing the raw legume, field bean (*Vicia faba*), as the major source of protein suffered significant reduction in growth rate and pancreatic hypertrophy. This study also agrees with that reported by Liener and Kakade (1980) that young rats and chicks fed raw soyabeans exhibited growth inhibition and pancreatic hypertrophy. This is primarily due to the presence in legumes of anti-nutritional factors which reduce food intake (Liener, 1981), impair digestion and absorption of dietary nitrogen (Nitsan and Liener, 1976), carbohydrate (Santidrian *et al.*, 1981) and lipid, (Sklan *et al.*, 1973) reduce the retention of observed nitrogen (Grant *et al.*, 1986) and elevate the catabolism of body lipid (Grant *et al.*, 1988), thereby limiting the efficiency of food utilization. Results of this study indicate that raw lablab beans contains very potent anti-nutritional principles since feeding the three varieties to rats led to the generation of oxidative stress and there was significantly depressed growth and feed consumption. Some of the pathological findings in this study are similar in several respects to those reported by King *et al.* (1980) for rats fed raw kidney bean, although no significant lesion was observed in the spleen, pancreas and intestine of rats fed the lablab beans. Some of the pathological findings observed in this study also agree with those reported by Aletor and Fetuga (1986), Aletor (1983), and Ologhobo (1980) who worked on lima beans and cowpea.

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Aletor and Fetuga (1988a) reported that growing rats and chicken fed diets containing raw field bean (*Vicia faba L.*) and soyabean (*Glycine max*) as the major source of protein suffered significant growth retardation, pancreatic hypertrophy and even death if the ingestion is prolonged. These deleterious effects have generally been attributed to the presence of a number of chemical substances such as trypsin inhibitors, phytohaemagglutinins, saponins, tannins (Eggum, 1980; Liener, 1980). Studies on growth performance in broiler chicks and rats showed adverse effects on body weight gain, feed intake or feed conversion ratios when tannin containing extracts were included in the diet (Ortalz *et al.*, 1994). Van Buren and Robinson, (1969) reported that tannins affect the growth of animals in three main ways. They have astringent taste, which affects palatability and decrease feed consumption, they form complexes with protein and reduce its digestibility and they act as enzyme inactivators. All these factors result from the interaction of tannins and proteins to form soluble and insoluble complexes, an interaction that depends primarily on relative proportion of phenol and protein.

The abundance of anti-nutritional and toxic influences of these factors within the lablab beans and most other edible legumes certainly calls for concern and therefore ways and means of eliminating or reducing their levels to the bearest minimum should be pursued. It could be wrongly argued that since our cultural method of preparing these legumes involves cooking, one needs not bother. This is not entirely correct because although the toxic effects of most anti-nutritional factors present in plant foodstuffs can generally be eliminated by proper heat treatment, it should be appreciated that conditions may prevail whereby complete destruction may not always be achieved. For example (Korte, 1972) observed that in mixtures of ground beans and ground cereals prepared under the field conditions prevailing in Africa, the haemaglutinin was not always completely destroyed, and that the cooked products produced diarrhoea and other toxicity signs.

A reduction in the boiling point of water in mountainous regions could also result in incomplete destruction of toxicity. The adverse effects of excessive cooking on protein denaturation is also an important consideration. Also, people don't patiently

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prepare or process beans to the recommended level to destroy the anti-nutrients because of the high and prohibitive cost of energy source like kerosene and gas and scarcity of firewood especially during the rainy season. As a result of this, people are forced to eat unproperly cooked nutritionally toxic beans (Pusztai *et al.*, 1993). The observation of biochemical changes is very relevant since some toxic substances take long periods to exhibit any symptoms or produce any organic damage. For example, symptoms of bracken poisoning may not appear until several months after the plant has been ingested. (Blood and Radostits, 1989). During the process of degeneration, leading to necrosis, several biochemical alterations occur. The changes in capacity of oxidative phosphorylation is one of the earliest manifestation measured by adenosine triphosphate (ATP) level (Beutler, 1969). Such an assault results in decreased intracellular pH and lack of energy for energy dependent cation pump working against normal electrochemical gradients.

Failure of the latter and loss of integrity of cell membrane results in an influx of sodium chloride, calcium and water leading to cellular swelling and leakage of intracellular ions (especially potassium), proteins and enzymes (Smith *et al.*, 1974 and Morgan *et al.*, 1988). These leaking enzymes provide an important diagnostic aid for the recognition of dead or dying tissues in living patients (Kaneko, 1989; Cornelius *et al.*, 1970). Measurements only allow for recognition of necrosis and by characterization of these enzymes by electrophoretic separation, the exact localization of necrosis to the specific tissue is said to be possible. Most studies dealing with the toxic properties of legumes had been mainly centered around those arising from intra-peritoneal (ip) injection of purified fractions with none or only one biochemical activity or feeding a purified fraction incorporated into a non-toxic diet (Apata, 1990). These experimental procedures may have different effects from that of a combination of toxic factors occurring and interacting naturally in the seed.

This is the rationale for the use of serum biochemical changes as one of the parameters for evaluating toxicity of the lablab beans in this study taking into consideration that the seeds were administered sub-acutely. Also, there is no sufficient literature in this part of the world that has used serum biochemical parameters as indices

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of toxicity for lablab beans and food legumes in general.All the authors did not include biochemical observations in their studies, as most studies reported only the gross and histopathologic effects of the seeds on major organs like the liver, kidney, pancrease, lungs etc. In this study, the three varieties of the raw lablab seeds caused a significant decrease in the level of packed cell volume (PCV), haemoglobin (Hb) concentration and red blood cell (RBC) count when compared with those of the control. The decrease was significant at $p>0.05$. The result of this effect on PCV, Hb concentration and RBC count showed that it has adverse effects on the erythron of rats (dysfunction of red cell haematopoiesis), and may therefore cause anaemia in animals that browse them. It is possible that the significant reduction may be the result of one or combination of the following toxic factors acting together in the raw beans to induce inhibition of haematopoiesis, a combined toxic factor-induced red blood cell haemolysis and an increase in the plasma volume (Apata, 1990). There was also an increase in the level of mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). There was not significantly different from that of the control and so the anaemia was normocytic anaemia. Extraneous poisons often reveal their toxic effects on the blood circulating system by numerous petechiae or ecchymoses, as a result of injury to the endothelium of capillaries, some haemolyze the circulating erythrocytes, a few destroy the haemopoietic powers of the bone marrow or the spleen whereas others produce their effect by blocking vital enzyme systems, usually without any identifiable lesion (Smith *et al.*, 1974; Beutler, 1969).

Poisonous plants may affect the formed elements of the blood by their actions on the production of blood in the bone marrow; by increasing their rate of peripheral destruction or haemolysis, or by influencing their distribution in various body portions. Anaemia can result from a lack of production of the various erythropoietic factors, inactivation of the factors (as by antibodies) or a failure of the bone marrow to respond to erythropoietin (Swenson, 1975). If damage to the bone marrow is severe enough, there may be an observed decrease in the numbers of the major groups of formed elements (pancytopaenia) (Jain, 1986). The observation of anaemia in this study supports the work of Seawright (1964) but is contrary to the work of (Sharma *et al.*,

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1982) who observed increases in the total RBC counts and PCV in *Lantana camara* poisoning.

The clinical implications of the production of anaemia by these *Lablab purpureus* seeds is quite obvious most especially in the system of livestock management in this country. With the transhumans in livestock management, the animals are constantly exposed to different plants which they are not used to and therefore are unable to differentiate between the poisonous and the non poisonous ones. moreso that the *Lablab purpureus* is newly introduced in to our environment as a source of animal feed. Tannins are soluble in water, dilute alkalis, alcohol etc but are generally only sparingly soluble in other organic solvents. Clinical signs, such as salivation, inappetence and ataxia of the hind limbs were noted in tannin poisoning of some plants like *Acacia decurrens* and *Acacia salicina* by (Clarke and Clarke, 1975). The tannin content of the lablab seeds may have contributed to the reduced PCV noted in this study. Saponins are reported to have pronounced haemolytic properties (Gee and Johnson, 1988). Different saponins have very different haemolytic activities, and there is a wide variation among different animal species in the susceptibility of their erythrocytes to haemolysis by saponins. Some of the soy saponins have little haemolytic activity (Birk *et al*., 1963; Lower, 1985). Saponins have the ability to rupture erythrocytes, and several authors have proposed to detect the presence of saponins in drugs, and to estimate their concentration, by measuring the haemolytic activity. Toxic plants do not produce a direct effect on the WBC, such as neutrophils, lymphocytes, eosinophils and monocytes (Swenson and Reece, 1993). However, excessive ingestion of a wide variety of plants has been found to cause hypoproliferative or non-regenerative anaemia which is a stem cell disorder characterized by reduced bone marrow production of all blood components in the absence of a primary disease process infiltrating the bone marrow or suppressing haematopoiesis (Kingsburry, 1964; Olsen *et al*., 1984). There was a decrease in the total white blood cell count (TWBC) relative to that of the control rats.Decrease in WBC count reflects a decline in the production of defensive mechanisms to combat infections. This situation will normally make the rats more susceptible to various physiological and biochemical stress resulting in disease, greater mortality and poor growth as reported by

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(Agrawal and Mahajan, 1980). There is also a reduction in the level of circulating lymphocytes as compared with the control rats. A decrease in the lymphocyte level is associated with conditions such as irradiation or corticosteroid administration. Increased concentration of corticosteroid is reported to cause lymphoid and thymic atrophy (Esteban, 1968). It thus means that continous administration or exposure of animals to these seeds is dangerous because the immune system of the body could easily be affected or compromised. Apata (1990) reported increase in lymphocytes in rats fed some tropical legume seeds like African yam bean and pigeon pea. It was stated that this may be due to stimulation of the reticuloendothelial system by the potent endogenous toxic substances in these legumes. It has been reported that haemagglutinins from mature and immature seeds affect lymphocyte stimulation (Ikegwuonu and Bassir, 1977; Lis and Sharon, 1977). The decrease noticed in the TWBC may be due to the increased level of circulating neutrophils occasioned by the feeding of these legumes in the raw form.

In general, the changes in the haematological parameters accompanying the ingestion of raw legumes appear to reflect degradation of the blood cellular constituents induced by the combined toxic principles in the intact beans. Toxic substances from poisonous plants affect the cellular elements of blood by causing direct haemolysis of the circulating erythrocytes (Casarett and Doull, 1975).

It is, however, important to note that several haematological traits vary in a known way in a wide range of nutrition and pathological situations. Therefore, some enzymes in serum have been employed in this study to provide additional information necessary to fully evaluate the toxicities of these legumes. Variation in the concentration of certain enzymes as measured by their biochemical activity, occurs primarily as a result of elevation due to the escape of the enzymes from the disrupted parenchyma cells with necrosis or altered membrane permeability. Our results also confirm those of Sathyamoorthy *et al.* (1981) and Aletor and Fetuga (1984b) who found that the serum ALT and AST in the rat were significantly increased when fed raw green gram and lima based diets. Increased activities of serum ALT and AST are indicative of increased catabolism of amino acids, since the activities were found to be markedly elevated and a

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concomitant increase in the serum urea is evident.Also, in terms of physiological response, the rise in AST level is symptomatic of liver cell damage. Thus the high AST levels in rats maintained on lablab beans could therefore be predictive of liver injury which might be attributed in part to the toxic effiects of some of the determined and undetermined factors on the cells of the organs. Aletor and Fetuga, (1985) have suggested that haemagglutinin could impart strong stimulus oo the liver cells, thereby allowing the enzymes to escape into the extra hepatic fluids.

Alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) are two membrane associated enzymes. They were significantly increased in all the rats fed the three varieties of the lablab beans. Elevation of serum ALP would mean that the hydrolysis of monophosphoric esters in the liver cell is concomitantly elevated (Medway *et al.*, 1969). Increases in serum ALP activity signals liver damage (Rosalki and Wilkiron, 1976). GGT is a glutathione-degrading enzyme, it is involved in mobilizing cysteine from the liver glutathione pool. Increased activities of serum GGT are suggestive of increased amino acid catabolism due to raw legume poisoning (Apata, 1990). An increase in serum concentration of GGT may indicate myocardial infarction (Hood *et al.*, 1990), congestive cardiac failure and liver cancer (Stark, 1991). Results of GGT could not be compared with other published work because it has not been monitored in previous experimental animals fed raw legumes. However, the levels of GGT were found to be significantly increased along with the liver specific enzymes (AST and ALT). Thus GGT may be used as an effictive tool for assessing hepatotoxicity.

Serum urea and creatinine levels were significantly elevated in all groups of rats fed the raw lablab beans, while the serum total proteins and albumin levels decreased. Our results on serum urea and creatinine agree with Sathyamoorthy *et al.* (1981) who reported increase in serum urea and creatinine in rats fed raw green gram. These authors also showed that increased activities of amino acid-degrading enzymes. arginase and ornithine transcarbamoylase bring about elevated levels of urea and creatinine. The increased urea and creatinine levels observed in this study must have been mediated through the effects of endogenous toxic substances, that probably reduce protein

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utilization thereby increasing the catabolism of amino acids which were subsequently degraded into urea and creatinine. Increase in blood urea in poisoning situation has been attributed to decrease renal excretion associated with organic disease of the kidneys with destruction of a considerable portion of functional renal tissue. Glomerulonephritis is a common cause of abnormally high blood urea nitrogen which is an evidence of renal functional impairment in acute and chronic glomerulonephritis (Abatan, 1992). Renal conditions accompanied by marked oliguria or anuria, lower nephron nephrosis are associated with increase blood urea (DeBruine, 1976). As urea is normally formed in the liver, decrease blood urea nitrogen are observed in conditions associated with acute hepatic insufficiency (Cantarow and Trumpert, 1956). Increase in blood urea in rats fed *L. purpureus* seeds is an indication that the urea formed in the rats was not being adequately excreted as a result of renal insufficiency.

Serum proteins are a form of storage amino acids. Enhancement of serum proteins imply a rise in amino acid absorption and utilization (Apata, 1990). The reduced serum proteins and albumin levels manifest an alteration of normal systemic protein utilization. This alteration can be attributed to an interference in protein synthesis (Apata, 1990). Factors responsible for decreased level of serum protein include toxic liver deficiency (Cantarow and Trumpert, 1956; Kaneko, 1989). This result from defective protein manufacture in the liver. The response either arise from the direct effects of hepatotoxicants on the kupfer cells or are secondary to hepatocellular insult. Albumin is said to notably decrease though hypoalbuminaemia may also follow upon excessive loss of protein into the urine in case of severe kidney damage (Abatan, 1992). The decreases observed in the serum protein indicate that *L. purpureus* seeds produced hepatocellular toxic changes and or nephrosis. Hypoalbuminaemia is reported to be most consistently demonstrable in acute and subacute hepatic nephrosis (Kaneko, 1989). The liver is the organ chiefly if not entirely responsible for the formation of the plasma albumin. It would therefore be anticipated that hepatic function impairment might result in decrease in the plasma albumin concentration. Since the globulin levels in the rats fed with the raw lablab seeds did not show significant changes, it can be inferred that the decrease in serum total protein in these animals is as a result in their reduced serum

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albumin levels. It has been observed that in some liver damages, the level of total protein is increased as a result of increased serum globulin level to offset the hypoalbuminaemia that occurs (Cantarow and Trumpert, 1956; Kaneko, 1989). In some cases, it is even reported that the total protein concentration may not reflect either the nature or the extent of an existing abnormality and may indeed even fail to indicate presence. This is as a result of changes in the albumin-globulin fractions in opposite directions to offset each other (De Bruine, 1976). The work presented here indicates that the toxic effects which accompany the feeding to the rat of raw legumes studied, derive in part from low feed intake, impaired protein utilization, reduced absorption of dietary components coupled with alterations in blood cellular components and activities of some serum enzymes.

Elevation in the activity of AST can be associated with cell necrosis of many tissues (Kaneko, 1989). For example, pathology involving the skeletal or cardiac muscles and or the hepatic parenchyma allows for the leakage of large amounts of this enzyme into the blood (Kaneko, 1989). The elevation in AST produced by this seed is an indication of tissue necrosis (Kaneko, 1989). ALT, on the other hand is present in liver and other cells. It is particularly useful in measuring hepatic necrosis, especially in small animals (Cornelius, 1989). Since, it is one of the specific liver enzymes assayed, its elevation in this study may indicate hepatic damage by the lablab seeds, although elevations in serum activity profile provides little information regarding the type of lesion or functional state of an organ (Kaneko, 1989), it can be used to gain a quantitative estimate of the extent of necrosis. Enzymes which increases in concentration in the blood following hepatic necrosis are divided into two groups: enzymes which are liver specific in that high concentrations are present primarily in hepatic tissue such as ALT (SGPT) and enzymes which are high in concentration in other tissues in addition to the liver such as AST (SGOT), lactate dehydrogenase and serum isocitric dehydrogenase. Moreover, the activities of the liver specific enzymes are the most sensitive and reliable test available for detecting mild to severe hepatic necrosis. AST is present in plasma cells. Acute hepatic diseases causing membrane damage or cell necrosis result in appreciable increase in plasma activity of the enzyme.

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ALP is reported to be present in a large number of cells but only in a few is the activity sufficient to be of clinical importance. In general, ALP activity is associated with the microvilli of secretory and absorptive cells such as epithelium of bile duct canaliculus, intestinal tract, renal tubular epithelium and placenta. It is also found in liver cells and in association with osteoblastic activity in the bone (Hoffman *et al.*, 1977; Hoffman and Dorner, 1977 and Saini and Saini, 1978). When obstruction of duct system occurs at any level in the liver, there is increase in hepatic ALP activity in serum (Hoffman *et al.*, 1977). Liver ALP activity is also reported to increase in hepatic fibrosis produced by poisonous substances. Increases in the serum activities of ALP associated with the Rongai brown variety of *Lablab purpureus* can be adduced to be most likely associated with the microvilli of absorptive or secretory cells in the rats. Sharma *et al.* (1982) also reported elevated levels of ALP in guinea pig poisoned with *Lantana camara*. In their clinico-pathologic features associated with *Aspergillus flavus* poisoning in pigs, other authors reported the observation of elevated levels of ALP among other serum enzymes and concluded that the increased levels of the enzymes is related to the hepatic damage caused by the plant (Kaneko, 1989). The principal lesions occur in the liver and they conclude that it can be classified as toxic hepatitis. Similarly, increases in the serum alkaline phosphatase were observed in goats poisoned with *Dichapetalum madagascariense* and *Lantana camara*. It has been reported that poisonous plants could produce destructive lesions in the liver (Doerr *et al.*, 1976).

Duncan *et al.* 1994 also reported that elevation in AST and ALT levels may suggest the hepatotoxic effects of toxic plants on rats. Kaneko, (1989) also reported that increased level of serum ALT and AST may suggest that such plants could produce a toxic effect on tissues and other organs in the animal body. *Lantana toxicity* in guinea pig is said to cause an elevation in AST activities (Sharma *et al.*, 1981). The histopathologic changes associated with Rongai brown are on the liver, kidney, spleen and testes but mild lesions or no significant lesions were observed in most of the other organs. The necrotic lesions produced in the liver may indicate systemic disturbances. Similar elevations in AST activities have also been observed in some plant poisoning such as with members of Senecio (ragworths) (Ford *et al.*, 1968); in calves; aflatoxicosis

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in swine, Lantana toxicity in guinea pig (Sharma *et al.*, 1981). All these observations were associated with tissue damages such as liver damage as well as nephrotoxicity.

The problem of poisonous plants is not their direct toxicity to man and animals alone but also the inconvenience and economic loss associated with the poisoning of domestic animals and the cost of preventing or reducing such happenings. It is worthy of note that plant poisons can either be accumulated in the animal or in certain organs or they are metabolized and excreted in milk (Liener, 1969). Ruminants may convert cyanide to the less toxic thiocyanate, which is goitrogenic (Jones *et al.*, 1997). By this food chain, toxins or their metabolites thereof may become harmfull to man (Habermehl, 1987). The haematological and biochemical aberrations induced in rats by feeding raw lablab bean in this study indicated the extent of toxicity of these seeds when induced in animal diets.

5.6 Studies on Antioxidant and Free radical scavenging activities

The weight loss in the rats fed the three varieties of lablab beans (Table 15) could be as a result of the reduction in feed intake or reduced absorption and utilization of nutrients due to the antinutritional factors (ANF) present in the beans. The decrease in testes weight of the rats fed the lablab beans may also reflect the toxicity of the ANF on testicular function. ANF are reported to reduce feed intake and nutrient utilization/nutritional quality of plant foods (Osagie, 1998). There were significant decreases ($p<0.05$) in the protein level of liver, kidney and testes of rats fed the three varieties of labalab beans except in the liver of rats fed the Rongai brown variety and the testes of rats fed the Rongai brown and Highworth black varieties, in which the decrease was not significant, (Table 16).

Histological examination of the liver of rats fed Rongai brown variety of lablab beans showed central venous and portal congestion, diffuse vacuolar degeneration of hepatocytes and mild mononuclear cell infilteration, the liver of rats fed Rongai white variety showed very mild portal cellular infilteration and severe periportal cellular infilteration by mononuclear cells while the liver of rats fed Highworth black variety showed multiple areas of cellular infilteration and mild hepatic necrosis while no visible lesions were found in the liver of rats in the control group. Also, no visible lesions were

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found in the kidney and testes of the rats fed the three varieties of lablab beans including the rats in the control group. The lesions found in the lever of rats fed the three varieties of lablab beans could imply that the ANF induced severe changes in the organ forms.

There were significant increases ($p<0.05$) in lipid peroxidation (LPO) in all the organs (liver, kidney and testes) of all the rats fed the three varieties of *lablab purpureus* (lablab beans) seeds, (Table 17). LPO is a degenerative mechanism of membrane component mediated through free radical production in the cell (Veena *et al.*, 2007). Increased level of LPO implies membrane instability which correlates with altered tissue membrane as is observed in the present study. Oxidative stress occurs in cells or tissues when the concentration of reactive oxygen species (ROS) generated exceeds the antioxidant capability of that cell (Trush *et al.*, 1991). The antioxidant enzymes play an effective role in protecting the testes and other biological tissues below a critical threshold of ROS, thus preventing testicular dysfunction (Oschsendorf, 1999). The antioxidant systems are a mutually supportive team of defence against ROS in reproductive organs (testes and epididymis) and other biological tissues (kidney and liver). LPO is the most extensively studied manifestation of oxygen activation in biology. LPO is broadly defined as "oxidative deterioration of polyunsaturated fatty acid (PUFA)" which are fatty acids that contain more than two cation double bonds (Halliwell, 1990). In general, the most significant effect of LPO in all cells is the perturbation of membrane (cellular or organellar) structure and function (transport processes, maintenance of ion and metabolite gradients, receptor mediated signal transduction, etc). Spermatozoa, unlike other cells, are unique in structure, function, and susceptibility to damage by LPO (Alvarez *et al.*, 1987). Spermatozoa are unable to repair the damage induced by excessive ROS because they lack the cytoplasmic enzyme systems that are required to accomplish this repair. This is one of the features that make spermatozoa unique in theirsusceptibility to oxidative insult (Krauz *et al.*, 1994).

Studies on how these cellular changes caused by LPO affect seminal parameters and sperm function and reversal of these effects are open to further investigations (Maneesh and Jayalekshmi, 2006). Catalase and peroxidase protects superoxide dismutase (SOD) against Inactivation by H_2O_2. SOD reciprocally protects catalase and

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peroxidase against inhibition by superoxide anion and peroxides generated in the testes, epididymis, liver and epididymis. Significant increases ($p<0.05$) in the catalase activity of all the organs (liver, kidney and testes) of rats fed the lablab beans, (Table 18), may reflect the ability of the enzyme in these organs to eliminate the H_2O_2 produced by excessive generation of ROS. There were significant increases ($p<0.05$) in the SOD activity of liver of rats fed the Rongai white and Highworth black variety and in the kidney of the rats fed Rongai brown variety of lablab beans, (Table 19). An increase in SOD activity has been reported to be beneficial in the event of free radical generation (Yen *et al.*, 1996). However, a simultaneous increase in catalase activity is essential for an overall beneficial effect of an increase in SOD activity (Engelman *et al.*, 1995; Schaefer *et al.*, 1998). There were decreases in the SOD activity of kidneys of rats fed Rongai white and Highworth black variety. This finding is similar to the report of Virginia *et al.*, (2009) on sulfasalazine-induced oxidative stress. SOD protects spermatozoa against spontaneous O_2 toxicity and lipid peroxidation (LPO). SOD and catalase also remove (O_2^-) generated by NADPH-oxidase in neutrophils and may play an important role in decreasing lipid peroxidation (LPO) and protecting spermatozoa during genito-urinary inflammation (Aitken *et al.*, 1995).

The level of glutathione-S-transferase (GST) increased in all the three organs tested, liver, kidney and testes, but only the testes produced a significant increase ($p<0.05$), (Table 22). Glutathione is the most abundant antioxidant in the body. It plays an important role in protecting lipids, proteins and nucleic acids against oxidative damage. It combines with vitamin E and selenium to form glutathione peroxidase (Lenzi *et al.*, 1993). Also, the level of glutathione peroxidase (GPx) increased significantly ($p<0.05$) in all the organs, liver, kidney and testes, except for the rats fed the Rongai brown variety (Table 21).

Reduced glutathione (GSH) has a wide distribution in body tissues and it performs several functions, the most fundamental of which is protection of cells against lipid peroxidation. It achieves this by reducing H_2O_2 in a reaction catalysed by glutathione peroxidase (Knapen, 1999). In the present study, there were significant increases ($p<0.05$) in GSH levels of the kidney of rats fed Rongai brown variety of

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lablab beans and that of testes of the rats fed Rongai white and Highworth black variety (Table 20). GSH is one of the most important non-enzymatic anti-oxidant against cellular damage produced by ROS (Luberda, 2005). In addition, significant functions of GSH in spermatogenesis and the reproductive process have been reported (Kaneko *et al.*, 2002). The increase in GSH levels observed in this study may be as a result of the body system trying to mop up the free radicals generated by the ANF in these organs. There were significant decreases ($P<0.05$) in the GSH level of the liver of all the rats fed the three varieties of lablab beans (Table 20). These alterations in GSH reflect a state of imbalance in the antioxidant systems in these organs which make it difficult to combat the ROS that is generated as a result of the ANF/toxic factors in the lablab beans, thereby leading to increased peroxidation of the membrane of these organs. GSH is also (glutathione-S-transferase) GST co-substrate (Virginia *et al.*, 2009). GST catalyzes the addition of GSH to a wide variety of endogenous compounds and xenobiotics (Romeve *et al.*, 2002). GST activity was increased in all the organs (liver, kidney and testes) of all the rats fed the three varieties of lablab beans although the increase was only significant ($p<0.05$) in the testes of rats fed the Rongai brown and Rongai white variety of lablab beans.

The alteration observed in the GST activity could be as a result of free radical activation/inactivation of this detoxifying enzyme. With respect to the significant increase produced in the testes, this could be due to the fact that testes are more sensitive to oxidative stress (Donnely *et al.*, 1999; Saleh and Agarwal, 2002). There were increases in H_2O_2 generation of all the organs of the rats fed the lablab beans, (Table 23), although the increase was only significant ($P<0.05$) in the liver of the rats fed Rongai white and Highworth black varieties. The increase in H_2O_2 generation observed may be a reflection of oxidative stress induced by the antinutritional factors on these organs. Testes and sperm function are particularly vulnerable to the injury produced by ROS (Shen and Sangiah, 1995; Kara *et al.*, 2007). It has been reported that hydrogen peroxide (H_2O_2), a potent oxidant, inhibits steroidogenesis in leydig cells (Diemer *et al.*, 2003). An increase in oxidative stress causes ROS-induced damage to macromolecules such as DNA, protein and key enzymes involved in testicular steroidogenesis and

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spermatogenesis (Sen *et al.*, 2004). During spermatogenesis and spermiogenesis, many macromolecules are synthesized. Sertoli cells secrete both serum proteins and testes specific proteins including androgen binding protein, inhibit sertoli cell derived growth factors and cyclic protein-2. Nair and Verma (2000) reported that the reduction in the synthesis of macromolecules could be responsible for the reduction in spermatogenesis and spermiogenesis. It is likely that the reduction of spermatozoa in the rats fed the three varieties of lablab beans (Table 24) is a manifestation of decreased spermatogenesis. The significant decreases ($p<0.05$) in sperm motility, viability, testicular sperm number (TSN), epididymal sperm number (ESN) and testes weight and the significant increases ($p<0.05$) in percentage (%) abnormality of rats fed the three varieties of lablab beans, as compared to control rats, could indicate that the anti-nutritional factors (ANF) present in them severely impaired spermatogenesis. Free radical-induced oxidative damage to spermatozoa is a condition, which is recently gaining a considerable attention for its role in inducing poor sperm function (Maneesh and Jayalekshmi, 2006).

Higher levels of ROS were correlated with a decreased number of motile sperm and conversely greater sperm motility was observed in samples with low amounts of detectable ROS (Iwasaki and Gagnon, 1992). Studies by Ollero *et al.* (2001) and Gil-Guzman *et al.* (2001) have shown that levels of ROS production in semen were negatively correlated with the percentage of normal sperm forms as determined by the (WHO, 1999) classification and by the strict criteria of Kruger *et al.* (1987). There was significant variation in levels of ROS production in subsets of spermatozoa at different stages of development. ROS production was found to be highest in the immature sperm fraction (containing sperm with abnormal head morphology and cytoplasmic retention) and lowest in the mature sperm fraction (containing normal-looking motile sperm) and in immature germ cells.

Reactive oxygen species (ROS) must be continuously inactivated to keep only a small amount necessary to maintain normal cell function. Seminal plasma is endowed with an array of antioxidants to protect spermatozoa against oxidants (Alvarez *et al.*, 1995; Sikka, 1996; Armstrong *et al.*, 1998). Antioxidants, in general, are compounds and reactions, which dispose, scavenge and suppress the formation of ROS, or oppose

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their actions. Among the well-known biological antioxidants, superoxide dismutase (SOD) and catalase play a significant role. Increases in percentage of sperm abnormalities have most frequently been observed as one of the ealiest indicators of testicular pathology (Noakes *et al.*, 2001). The motility of sperm cells is important for fertilization to occur since only sperm cells with progressive motility are involved. The reduction in spermatozoa quality may be subject to the degenerative changes caused by the ANF/toxic factors and the lesions may affect several organs (Ajayi *et al.*, 2004). Hence, the increase observed in the percentage sperm abnormalities in the rats fed the three varieties of lablab beans could be said to be due to the ANF/toxic factors in the lablab beans. Toxic factors are reported to disrupt spermatogenesis (Kasinathan *et al.*, 1972). It has also been reported by Seth *et al.* (1981) that toxic agents could produce a dose dependent effect on the weight of the testes and significant reduction in sperm motility. It is however possible that once the exposure is discontinued, there may be gradual improvement/reduction in sperm abnormalities with complete testicular healing occurring some later time (Ahmed *et al.*, 2002).

Research during the last decade implicated oxidative stress as a mediator of sperm cells dysfunction (Sharma and Agarwal, 1996). It has been suggested that this phenomenon was related to the ability of sperm germ cells to generate reactive oxygen species (ROS). Spermatozoa are sensitive to OS because they lack cytoplasmic defenses (Donelly *et al.*, 1999; Saleh and Agarwal, 2002). Moreover, the sperm plasma membrane contains lipids in the form of polyunsaturated fatty acids, which are vulnerable to attack by ROS. ROS, in the presence of polyunsaturated fatty acids, triggers a chain of chemical reactions called lipid peroxidation (Kobayashi *et al.*, 2001; Zalata *et al.*, 2004). ROS can also damage DNA by causing deletions, mutations, and other lethal genetic effects (Moustafa *et al.*, 2004; Tominaga *et al.*, 2004). Spermatozoa are liable to be under a continous influence of OS because of excessive generation of ROS (Agarwal *et al.*, 2005).

Zemjanis (1970) reported that spermatozoa abnormalities such as headpiece abnormalities are considered to reflect disturbances of spermatogenesis while secondary abnormalities such as bent-tails, abnormal acrosomes are believed to arise after

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spermatogenesis is completed and as such due to epididymal dysfunction. Thus, the increase in percentage (%) level of abnormal sperms in the rats fed the three varieties of lablab beans (Table 24) could be as a result of testicular dysfunction. However, since the development of abnormal sperm head is reported to be polygenically controlled (Wyrobeck *et al*., 1983), the sperm head abnormalities observed in this study reflects the possible genotoxic effect of ANF or other toxic factors in lablab beans. The volume of ejaculate, colour and density, gross motility and presence of foreign material determines the quality of sperm. The motility test is considered to provide the most significant information about the quality of semen. It has been reported that at least 70% of the motile sperm cell should exhibit the progressive type of motility, while gross motility reflects the concentration and viability of sperm cells (Blom, 1948). Mammalian spermatozoa are rich in polyunsaturated fatty acids, and, thus, are very susceptible to ROS attack which results in a decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased midpiece morphology defects with deleterious effects on sperm capacitation and acrosome reaction (Lenzi *et al*., 1993). Lipid peroxidation of sperm membrane is considered to be the key mechanism of this ROS-induced sperm damage leading to infertility (Agarwal *et al*., 1994b).

The oxidant effect of ANF-induced reproductive toxicity is evident in sperm abnormalities such as reduced % motility, of sperm, reduced viability, reduced TSN and ESN, reduced testes weight and increased % abnormality, live-dead counts and sperm physiology and morphology. In normal circumstances, there is equilibrium between the generation of ROS and antioxidant strategies of the male reproductive tract, leaving only a critical amount of ROS required for normal sperm function. Excessive production of ROS, however, results in destruction of the antioxidant capacity of spermatozoa and seminal plasma causing oxidative stress which damages spermatozoa membrane and causes infertility (Lewis *et al*., 1995) and a number of lines of evidences have been presented indicating that oxidative stress plays a key role in the aetiology of poor semen quality and male factor infertility (Aitken, 1991).

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5.7 Molecular Weight determination of Lablab seeds proteins

The results of the electrophoretic studies obtained in this investigation support the heterogenous nature of lablab seed protein preparations. In this study, Rongai brown variety has the highest number of bands and hence proteins having 17 bands, followed by Rongai white having 15 bands and Highworth black recording the least with 12 bands. The observed differences in the protein patterns of the lablab seeds could be taken to reflect differences in the properties of the extracted proteins. The fact that some bands occurred in all or almost all the samples examined suggest that major differences, when they occurred, were real (Fafunso, 1972). (Wrigley *et al.*, 1966; Pirie, 1966a,e; 1970a,b.) reported that observed differences in protein patterns of leaf proteins could be taken to reflect differences in the properties of the extracted proteins. In the present study, twelve (12), fifteen (15), and seventeen (17) bands, representing the protein fractions were obtained for the Highworth black, Rongai white and Rongai brown varieties of the lablab seeds respectively. Each of the three varieties of lablab seeds have some bands, representing proteins which are peculiar to them only (Tables 25 and 26) which are peculiar to them only.

The protein yields of plants are reported to vary widely with different species, different extraction methods, different stages of maturity and also soil conditions (Matai *et al.*, 1970a, b). Smirnova-Ikonnikova (1962) observed significant variations in the accumulation of proteins in the seeds of different varieties of cowpeas and soyabeans and suggested that the nature of these changes depend on specific peculiarities and growing conditions of the legumes. Swaminathan and Jain, (1973) also reported that differences in nutrient contents of legumes are dependent on variety and location. The bands which are found consistently i.e. those present in all the three varieties of the *L. purpureus* seeds may be those proteins that give the lablab beans its distinctive characteristics from other grain legumes like soybeans, cowpeas, kidney beans etc. Interest in the area of plant proteins is largely due to the diverse applications of these Proteins in various fields of agriculture, biochemistry, nutrition, medical sciences and modern molecular biology. Osborn, (1988) reported molecular weight ranges of 43,000 daltons to 54,000 daltons for *Phaseolus*. Sathe *et al.*, (1994) reported that tepary bean

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(*Phaseolus acutifolius var lactifolius*) major storage globulins was composed of three glycosylated polypeptides with estimated molecular weigbts of 49,500, 45,890, and 44,510 daltons.

Molecular Weight determination of the *Lablab purpureus (lablab beans)* seed proteins has brought to light the quantitative and qualitative variations in the seed proteins of the three varieties of lablab beans studied. Efforts should be devoted to conducting more research to extend both technical and practical knowledge about *Lablab purpureus* so as to achieve its full potential. This will help to improve the protein intake and thus the living conditions of people in countries where shortages of protein supply exist for human and animals. This being so, the fractions which occur consistently may be those proteins that give the seeds its distinctive characteristics e.g. enzymes of the photosynthetic apparatus. Polyacrylamide gels have higher resolving power as compared to starch gels (Chang and Steward, (1962) and Dockes, (1968). The protein yields of plants are reported to vary widely with different species, different extraction methods, different stages of maturity and also soil conditions (Matai *et al.*, 1970a, b). Investigations of the components of various bulk leaf proteins by electrophoresis on polyacrylamide gels (Steward *et al.*, 1965; Nye *et al.*, 1968), on starch gel and on filter paper all indicate that plant proteins are mixtures of many individual proteins (Pirie.1966a). Shapiro *et al.*, (1967) and Weber and Osborne, (1969) have shown that the separation of proteins by polyacrylamide gel electrophoresis in the presence of anionic detergents sodium dodecyl sulphate is dependent on the molecular weight of the polypeptide chains. A plot of the electrophoretic mobilities against the molecular weight of the protein gives a smooth curve. In this study, sodium dodecyl sulphate polyacylamide gel electrophoresis (SDS-PAGE) was used to study the molecular weights of the proteins in the lablab seed.

The proteins in the lablab seeds fall in a molecular weight range of 7,000 to 50,000.Proteins in the range of 28,000 to 43,000 is common to all the three varieties of the lablab seeds. Separation on acrylamide gel of the proteins in the lablab seeds yielded 12,15, and 17 bands supporting the assertion that plant proteins are a heterogenous mixture of proteins.16 to 21 bands have been reported by (Wrigley *et al.*, 1966; Stavely

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and Hanson, 1967; McMullan and Ebell, 1970) and 30 distinctly different bands by vanLoon and vanKamman, (1968) for various leaf protein samples. 8-11 bands were observed for the centrifuged leaf juices from the various vegetables like *Telfairia occidentalis, Celosia argentea* (green), *C.argentea* (red), *Solanum incanum, S. anomalum* and *Curcubita pepo* studied by (Ayalogu, 1978). Similar observations about the heterogenous nature of plant protein preparations have been made by (Mendiola and Akawaza, 1964; Wrigley *et al.*, 1966 and Fafunso, (1972) using starch gel electrophoretic techniques. Mendiola and Akawaza, (1964) observed 7 bands for rice leaf protein and Wrigley *et al.*, (1966) reported 15 bands for wheat leaf protein. Fafunso, (1972) reported 6 bands for leaf proteins from various vegetables and demonstrated the presence of albumins and globulins in these proteins. Davis, (1964) had observed a substantial increase in the resolving power of sieving gel matrices over other conventional forms of electrophoresis.

Polyacylamide gels have the advantage of using thin gels which allow faster separations, better defined bands, faster staining after separation and better staining efficiency. However, acrylamide is listed as a neurotoxin and should be handled carefully when making stock preparations. In sephadex gel filterations, the separation obtained is based on dimensional differences only whilst in polyacrylamide gel electrophoresis and starch gel electrophoresis, the separation is based on dimensional and charge differences. However, in polyacrylamide gel electrophoresis, there is controlled variation of gel pore size forthe purpose of increasing the resolution of ions based on electrophoretic differences and a concentration of the sample ions into a narrow zone prior to starting the electrophoretic separation.

Similarities were observed in the electrophoretic patterns of the various leaf proteins in this study, although the number of bands differed in some cases. The similarities in the electrophoretic patterns of the lablab seed proteins observed in this study as well as the similar observation by Fafunso, (1972) using starch gel electrophoretic method and Ayalogu, (1978) using sodium dodecyl sulphate gel electrophoresis is stricking as Coulson and Sims,(1965a,b) have observed differences to exist in the protein patterns of different varieties of wheat at different stages of growth.

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Plant protein concentrates are an excellent source of protein for supplementing other foods which are low in protein quality. Their usefulness in supplementing diets have been demonstrated by Waterlow, (1962), Doraiswamy *et al.*, (1969), Subba Rau and Singh, (1970), Olatubosun *et al.*,(1972), Oke and Umoh, (1974), and Omole *et al.*, (1976). Fafunso, (1972) studied the changes occurring in the protein fractions of six edible vegetables at different stages of growth. It was found that the leaf protein concentrates extracted from these vegetables were mixtures of individual proteins and comparison with human serum showed that leaf proteins contained albumin and globulin amongst other protein fractions. The leaf protein fractions were found to vary with increasing age of the plant and were unaffected by seasonal variation.

The theory and application of polyacrylamide gel electrophoresis for plant proteins and serum have been discussed by (Steward and Barber, 1964; Steward *et al.*, 1965). This technique has been applied to study the soluble proteins of conifer foliage, clover, tobacco and wheat leaves (Wrigley *et al.*, 1966; Stavely and Hanson, 1967; vanLoon and vanKamman, 1968 and McMullan and Ebell, 1970). 16 to 21 bands have been reported by these workers for the soluble leaf proteins.

Free and Satterlee, (1975) used this technique to separate the protein components of centrifuged alfalfa juice. They observed 7 protein bands for centrifuged alfalfa juice and 6 for dialysed centrifuged alfalfa juice supporting the theory that the majority of these proteins were water soluble. The majority of the proteins (93.7%) exhibited a molecular weight in the range of 25,000 to 60,000 while the remaining protein was estimated to have a molecular weight of 600,000. Although, it is evident that legume seeds are increasingly recognized as important crops, not only for animal feeding but also for protein extraction processing and human food uses, it also appears that except for soybean, applications are still limited and the protein composition must be improved if they are to become more suited to user requirements. Molecular approaches to improve the nutritional and functional properties of legume seed proteins should therefore be developed.

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CHAPTER SIX
CONCLUSION

6.1 Conclusion

These studies have not only provided details about the biochemical composition and nutritional characteristics of a relatively neglected tropical legume seed, they have also revealed their possible toxic effects when ingested in the raw state. Apart from being rich in essential nutrients, these legumes contained varying levels of several toxic factors that signify the possible antinutritional role they can play in the utilization of these grain legumes as both human food and animal feeds. Among the lablab bean varieties studied, Rongai brown variety is potentially the most toxic because it is the only variety that produced necrotic lesions in the liver and the one in which the rats died earlier in the preliminary toxicity study. Results of the dietary toxicities indicate that the toxic effects which accompany the feeding of raw legume seeds to rats derive in part from low feed intake, impaired protein utilization, reduced absorption of other dietary components coupled to distinct alterations in blood cellular constituents and activities of some serum enzymes. These alterations point to the fact that the seeds may have toxic effects on the tissues and their continuous administration may lead to toxicity especially if grazed by livestock. Although *Lablab purpureus* is newly introduced into Nigeria as a source of forage for livestock, however, the raw seeds are toxic and caution should therefore be exercised in their nutritional use. Measures should be taken to reduce the antinutritional and toxic factors in the plant through various processing methods like cooking, germination, soaking, heating before feeding to livestock and man. The results of the antioxidant and free radical scavenging activities showed that feeding raw lablab beans to male rats induced alterations in the hepatic, nephrotic and testicular antioxidant systems, impaired testicular function, spermatogenesis and epididymal sperm physiology and morphology and thus the adverse effect of antinutritional factors (ANF) on the liver, kidney, testes and epididymis have been observed.

Molecular Weight determination of the lalab seed proteins revealed that Rongai brown had the highest number of proteins, followed by Rongai white, while Highworth black had the least number of proteins.

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6.2 Contribution to Knowledge

1. The variation in the levels of crude protein, crude fibre, minerals and the antinutritional factors of the three varieties of *L. purpureus* seeds, a lesser known legume have been determined.

2. The histopathological effects of the Lablab beans varieties have been revealed in this study. The Rongai brown variety caused severe diffuse necrosis of hepatocytes and also caused degeneration and necrosis of germinal epithelium of the testes.

3. The *Lablab purpureus* seeds induced alterations in the hepatic, nephrotic and testicular antioxidant systems, impaired testicular and epididymal sperm function. This is a novel discovery on the effects of antinutritional factors on vital organs of the body.

4. The Molecular weights of the seed proteins in the three varieties of *L. purpureus* have been determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

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APPENDIX

Appendix 1: Standard curve for protein determination by Biuret method

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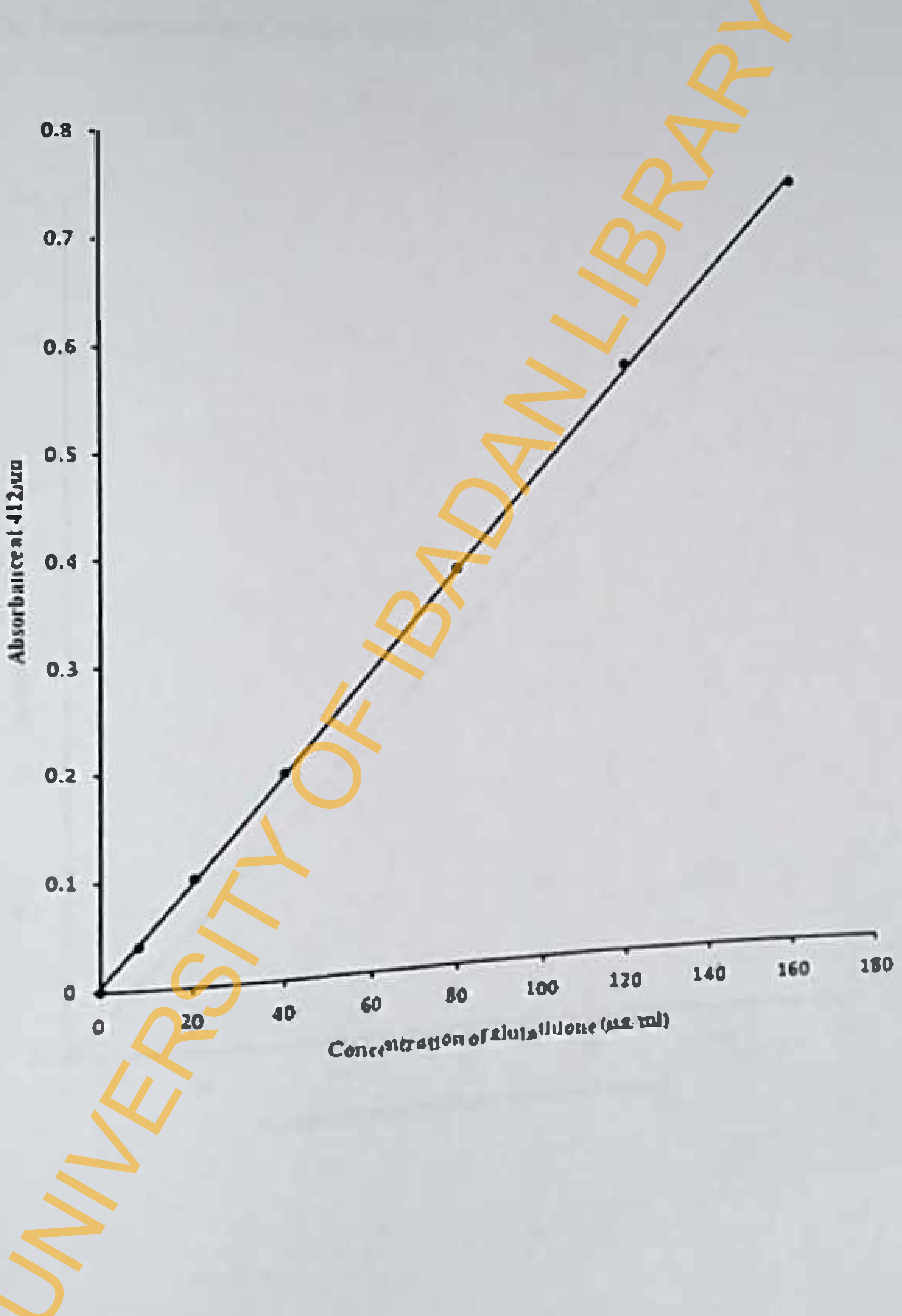
Appendix 2: Standard curve for reduced glutathione



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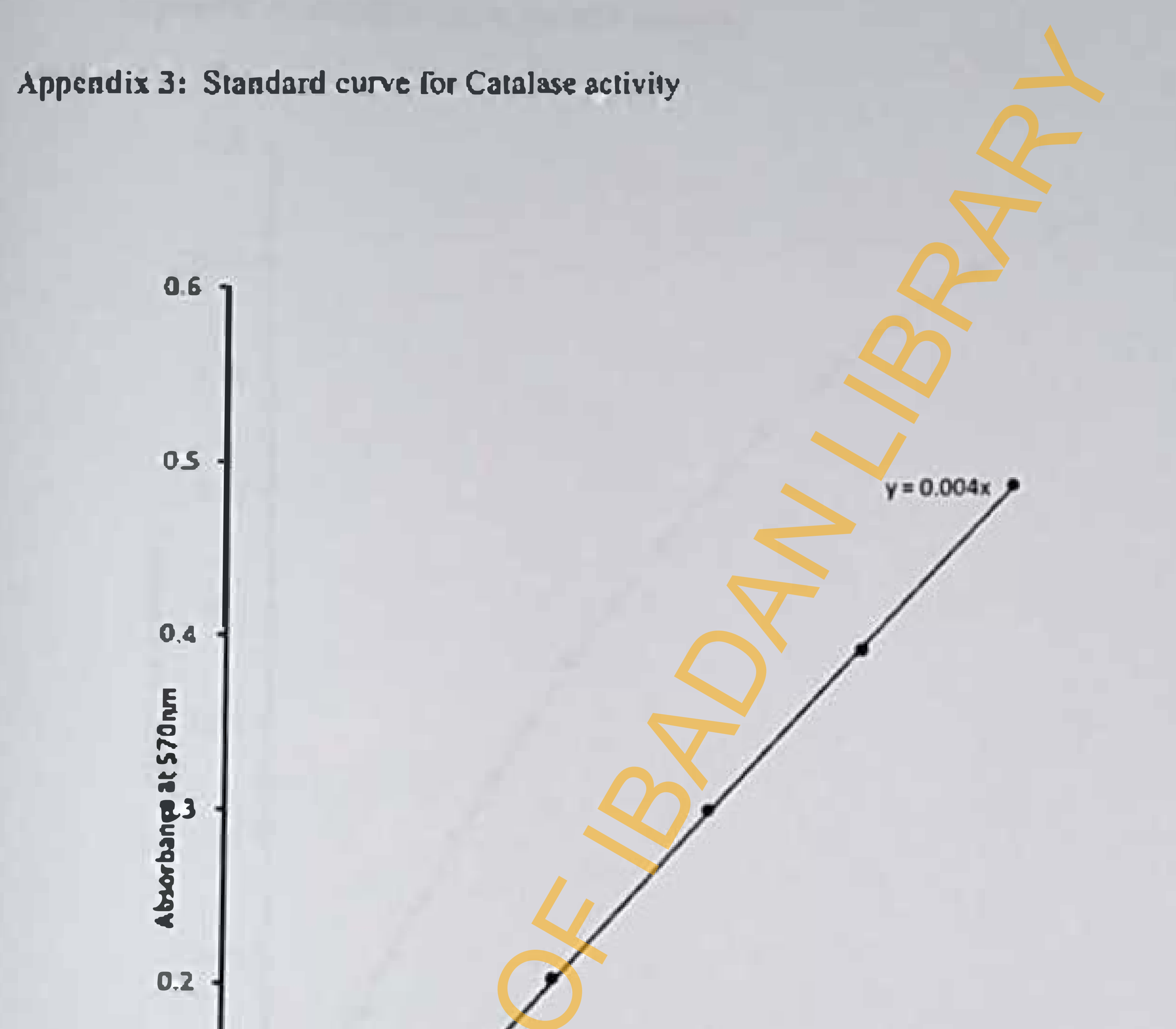
Appendix 3: Standard curve for Catalase activity



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Appendix 4: Standard curve for AST activities

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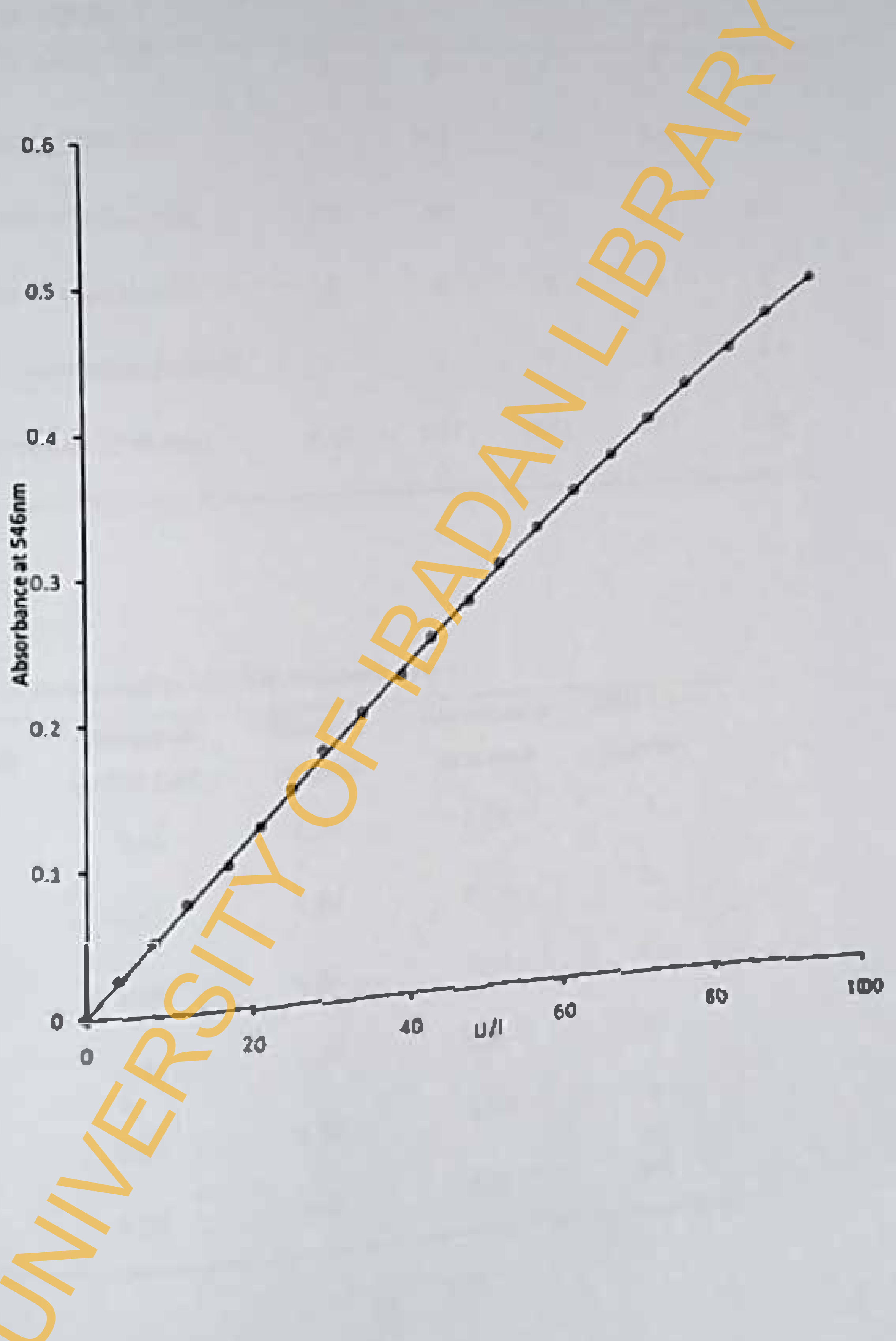
Appendix 5: Standard curve for ALT



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Appendix 6: Protocol for Protein estimation according to the method of Gornal *et al.* (1949)

Test tube No.	1	2	3	4	5
Stock BSA (ml)	0.1	0.2	0.3	0.4	0.5
Distilled water (ml)	0.9	0.8	0.7	0.6	0.5
Biuret reagent (ml)	4	4	4	4	4
BSA Concentration (mg/ml)	1	2	3	4	5
Absorbance (540 nm)	0.16	0.31	0.46	0.65	0.79

Appendix 7: Preparation of GSH standard curve

Stock (ml)	Phosphate buffer (ml)	Ellman's reagent	Absorbance (412nm)	GSH Conc. (µg/ml)
0.02	0.48	4.50	0.04	8
0.05	0.45	4.50	0.10	20
0.10	0.40	4.50	0.19	40
0.20	0.30	4.50	0.38	80
0.30	0.20	4.50	0.57	120
0.40	0.10	4.50	0.75	160

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Appendix 8: Protocol for the estimation of Hydrogen Peroxide

Test tube	1	2	3	4	5	6	7
H_2O_2 (ml)	0.05	0.10	0.15	0.20	0.30	0.40	0.5
Dichromate/ acetic acid (ml)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water (ml)	0.95	0.90	0.85	0.80	0.70	0.60	0.5
H_2O_2 concentrotion (μmoles)	10	20	30	40	60	80	100
Absorbance (570nm)	0.05	0.10	0.15	0.20	0.29	0.39	0.48

Appendix 9: Calibration of AST Standard Curve

Absorbance	U/l	Absorbance	U/l
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

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Appendix 10: Calibration of ALT Standard Curve

Absorbance	U/l	Absorbance	U/l
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

Appendix 11: Protocol for Urea Assay

	BLANK	STANDARD	SAMPLE
Serum Sample	-	-	10µl
Urea Standard	-	10µl	-
Distilled water	10µl	-	-
Reagent 1	100µl	100µl	100µl

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Appendix 12: Protocol for Creatinine Assay

	BLANK	STANDARD	SAMPLE
Distilled water	0.5ml	-	-
Creatinine Standard	-	0.5ml	-
TCA	0.5ml	0.5ml	-
Supernatant	-	-	1.0ml
Reagent Mixture	1.0ml	1.0ml	1.0ml

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